1-1-2013

Investigating the effect of corneal herpes simplex virus infection on toll like receptor expression in human peripheral blood mononuclear cells

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Investigating the effect of corneal Herpes Simplex Virus infection on Toll like receptor expression in human Peripheral Blood mononuclear cells.

This Thesis is submitted to the Royal College of Surgeons in Ireland for

the Degree of Master of Science (MSc)

RCSI

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November 2012

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Acknowledgements

This thesis would not have been possible without the help and support from many people who guided, prodded and pushed me in the right direction.

Thanks to my supervisors Dr. Caroline Jefferies and Professor Conor C. Murphy. All your support, guidance, reassurance and most importantly patience throughout the year have taught me a huge amount and I hope you find value in this thesis.

Thanks to Dr. David Shahnazaryan who recruited all the patients for the study, took their blood and helped me with any clinical aspects of the study that I was unsure about.

Thanks to Dr. Conor Malone who was the original procurer of funding for this project.

Thanks to all the Students, doctors and post docs in Lab 3a who showed me all the techniques, answered questions and put up with my lab tantrums when things were not quite going my way and made the year a very fun experience. To Siobhan, Rebecca, Kevin, Elisa, Jennifer, Barbara, Claire and Eoghan, your advice and guidance was invaluable and I truly appreciate all the time you took out to help me. A special shout out has to go to Dr Joan Ni Gabhann who, after no matter how many times I crowed JOAN! She patiently answered my questions no matter how daft.

To all my colleagues in RCSI who made the year easier with regular tea and coffee breaks. Thanks to Seamus, Jonathan, Annachiara, Naadiya, Tony, Kalyan, Tadhg, Lorraine and James.

To Emily, who listened to all my moaning and whinging and always brightened up my day. You are a legebag!!.

Thanks to my family. I've been blessed to be related to such a fantastic bunch of people and you always kept me sane when I felt I was about to tip over the edge.

Thanks to all the staff in RCSI and in MCT. To Kay and Olwen for all your help, it was much appreciated.

Thanks to my friends who I kind of abandoned and hopefully some of you are left when I finally get a life back.

Thanks to the Emerald Warriors who provided me with lots of fun nights and distractions from the grind of studying. You guys are Super!

Thanks to my Dad who provided many a dinner for a hungry Masters student.

Last but not least, thanks to my Mum who believed I could get through this even when I didn't.

Abbreviations

BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
CpG	Deoxycytidylate-phosphate-deoxyguanylate
DC	Dendritic Cell
dsDNA	Double stranded deoxyribonucleic acid
dsRNA	Double stranded ribonucleic acid
DTT	Dithiothreitol
ECL	Enhanced Chemiluminescence
ELISA	Enzyme-Linked Immunosorbent Assay
FACS	Fluorescence-Activated Cell Sorting
HSK	Herpes Simplex Keratitis
HSV	Herpes Simplex Virus
ICP	Infected Cell Protein
IFNα	Interferon alpha
IFNβ	Interferon beta
IFNγ	Interferon gamma
IgG	Immunoglobulin G
IKK	IκB Kinase
IL	Interleukin

IRF	Interferon Regulatory Factor
IκB	Inhibitor of kappa b
JAK	Janus Kinase
LPS	Lipopolysaccharide
MHC	Major Histocompatibility Complex
MYD88	Myeloid Differentiation Factor 88
NaCl	Sodium Chloride
NF-κB	Nuclear Factor κ-light-chain-enhancer of activated B cells
NK	Natural killer
ODN	Oligodeoxynucleotides
PAGE	Poly Acrylamide Gel Electrophoresis
PAMP	Pathogen-Associated Molecular Pattern
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
Poly I:C	Polyinosinic-polycytidylic acid
PVDF	Polyvinylidene Fluoride
RANTES	Regulated and Normal T cell Expressed and Secreted
RT	Room Temperature
SDS	Sodium Dodecyl Sulphate
SOCS	Suppressor of Cytokine Signalling

ssRNA	Single Stranded Ribonucleic Acid
STAT	Signal Transducer and Activator of Transcription
TAP	Transporter Associated with Antigen Processing
TBS	Tris Buffered Saline
TLR	Toll Like Receptor
TNF-α	Tumour Necrosis Factor alpha
vhs	Virion Host Shutoff protein

Abstract:

Herpes Simples Keratitis (HSK) is the commonest cause of infectious blindness in the developed world. It is caused by HSV-1 which is a large double stranded DNA virus which can invade the cornea and after treatment remains latent in the trigeminal ganglia. Toll like receptors are key components of the innate immune system and are highly expressed in the corneal epithelium. HSV-1 induces upregulation of several Toll Like Receptors (TLRs) and triggers the release of anti-viral cytokines. In certain cases HSV-1 has evolved to avoid these innate anti-viral responses and can cause lifelong recurrent infection. This recurrent keratitis causes lesions which are immunoinflammatory in nature, can recur throughout life and cause progressive corneal scarring, vascularisation, thinning and may require a corneal transplant which does not have a good long-term outcome. Understanding the mechanisms that cause this disease may lead to improved or novel therapies that will help the long-term outcome of HSK patients. This study aims to examine the immune responses of HSK patient peripheral blood mononuclear cells (PBMC) before and after treatment compared to healthy donors. Our work has shown that peripheral blood mononuclear cells (PBMCs) show differences in their ability to respond to various TLR ligands both in relation to the cytokines they produce and upregulation of cell surface markers. In particular our results show that IL-1β, a key proinflammatory cytokine is elevated in serum of active patients compared to inactive patients, thus demonstrating that peripheral immune responses are activated in response to HSV infection of the cornea. In addition our work has shown that although active patients expressed activation markers on T cells and B cells but differences were inconclusive based on patient numbers (active/inactive n=5, control n=4). Further data indicated that the TLR 3 pathway is compromised in active patient PBMCs, potentially having implications for HSV-1 viral clearance and viral replication and HSK persists in the cornea.

This work was supported by a grant from Pfizer and the Irish College of Ophthalmologists.

Investigating the Effect of Corneal Herpes Simplex Virus Infection on Toll Like Receptor Expression in Human Peripheral Blood Mononuclear Cells.

1. Introduction

1.1 Herpes Simplex Keratitis

Herpes Simplex Keratitis is a sight threatening infection affecting up to 90% of adult populations in certain countries. (Xu, Sternberg et al. 2006). It is caused by the herpes simplex virus type 1 (HSV-1) a double stranded DNA virus which is an ubiquitous pathogen in all global populations. Oral and genital infections are more common area of HSV-1 infection but ocular disease is also a serious health issue and can present inflammation and lesions on all major ocular tissues including the cornea. A major complication arising HSV-1 is that after primary infection the virus establishes a state of latency in the trigeminal ganglion nerve where it will remain as a lifelong latent infection. Depending on certain conditions the virus can undergo reactivation causing replication of the virus in the cornea and surrounding tissue. This cycle of latency and reactivation causes inflammation and scarring that can permanently damage the cornea which may require a corneal transplant which does not have a good long term outcome. The aim of this project is to examine the immune response of patients that have presented at the Royal Victoria Eye and Ear Clinic with active HSK as diagnosed by an ophthalmologist. Active disease is diagnosed when the eye is red, there is an ulcer or oedema of the corneal stroma with inflammation present in the anterior chamber of the eye. To examine differences blood was taken from patients at this active stage and during their second visit if they have been confirmed to have Inactive disease which is when all these symptoms have subsided. To determine any irregularities in the immune responses of these patients who suffer recurrent HSK episodes peripheral blood mononuclear cells (PBMC's) were isolated from the patients blood and subjected to a series of analytical tests to examine immune function.

1.2 Epidemiology

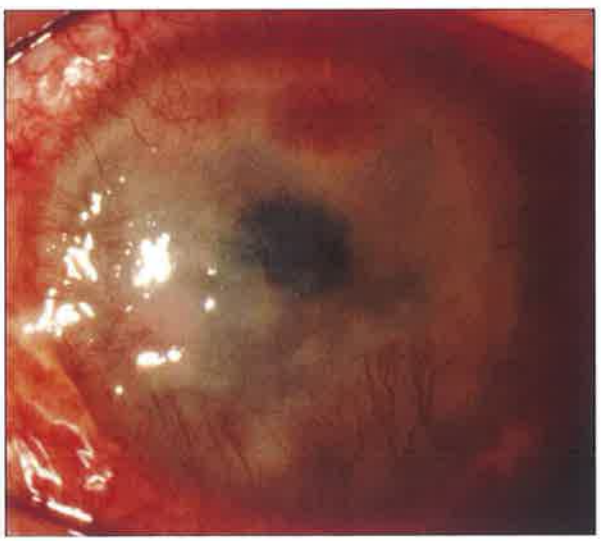
Depending on the population studied, the seroprevalence of HSV-1 can be as high as 90%. Indeed, 92% of individuals with no history of primary herpes infection have been shown to shed HSV-1 DNA in their tears (Leigh, Acharya et al. 2008). The majority of general adult populations globally carry the HSV-1 virus in its latent form and never presenting ocular disease. The incidence of herpetic ocular surface disease from HSV-1 lies between 5.9 per 100,000 and 20.7 per 100,000 with a prevalence of 149 per 100,000 in developed countries (Liesegang 2001). A study in the USA estimated that new ocular HSV infections were 11.4 per 100,000 which had risen from 8.4 two decades earlier (Liesegang 1989). A study in France identified an incidence of HSK of 31.5 per 100,000 population of which 13.2 were new cases and 18.3 were recurrences (Labetoulle, Auquier et al. 2005). Of the symptoms that were presented were epithelial dendritic lesions (56.3%) were the most common followed by herpes stromal keratitis (29.5%) (Labetoulle, Auquier et al. 2005) which is a more severe form of HSK. These studies show that HSK is a significant health issue amongst the general public due to the high risk of recurrence following a single episode of Keratitis. HSK is difficult to treat and current knowledge of the immune deficiencies causing these recurrences is poorly understood.

1.3 Clinical features

HSK occurs after the HSV-1 virus has caused either a primary infection through direct contact from the ocular surface or through translocation from another affected area, generally the mouth. Infection from direct contact of the eye with the HSV-1 virus termed the front door route or from a non-ocular site of infection e.g. mouth is termed the back door route of infection. (Tullo, Shimeld et al. 1982; Kaye and Baker 1996). The primary infection from HSV-1 usually occurs early in life and usually does not present with clinical symptoms(Umene and Sakaoka 1999). Manifestations of clinical disease in primary infection and recurrent HSK include conjunctivitis and inflammation around the eye including the eyelid. Ulcers can form as well as dendritic lesions in the epithelium of the cornea (Darougar, Wishart et al. 1985). The lesions caused by HSV 1 can either affect the epithelium alone or affect the stromal without damage to the overlying epithelium which can be

termed either simplex keratitis or stromal keratitis. The lesions in the epithelium are caused by the HSV virus replicating in the epithelial cells and destroying them (Liesegang 1999).These lesions are controlled by the immune system and have been shown to heal more rapidly when treated with antiviral drugs (Porter, Patterson et al. 1990). Other clinical signs of HSK in the human eye include stromal opacity, oedema and neovascularisation that can occur due to frequent outbreaks of the HSV virus (Kumaraguru, Davis et al. 1999). Due to complications that can arise from recurrent episodes of HSK which can lead to corneal damage and visual morbidity and also in the severe necrotising form of the disease, can cause corneal melting. Therefore there is a need to develop better treatments that can both treat the infection quickly to limit the damage caused by replicating virus and also prevent the reactivation of the disease and keep it in its latent form. Corneal transplants for HSK can fail for a number of reasons. Trigeminal neurons that are severed during the removal of the cornea can result in HSV transport to the graft site. Even if the corneal transplant is initially successful the chance of recurrent HSK is high and eventual destruction of the donor cornea. A recently developed procedure named deep anterior lamellar keratoplasty (DALK) which transplants the corneal stroma whilst retaining the host endothelium may prove to be more successful, as damaged endothelium is a common source of transplant rejection.

A



B

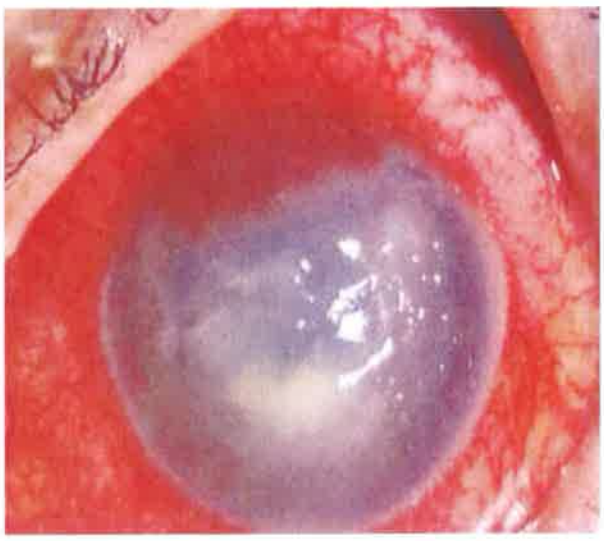


Fig 1: Clinical features of HSK showing A: neovascularisation , necrosis, Corneal thinning B: corneal scarring and opacity.

1.4 HSV-1 and the Immune system

The innate immune response is mostly responsible for the clearance of the HSV1 virus following primary infection with a lot of evidence that Natural Killer (NK) cells and macrophages are recruited to the eye soon after HSV1 infection (Frank, Buela et al. 2012). Little is known about the combined effect of adaptive and innate immune response on viral clearance although it has been shown that mice with no adaptive immune system but a fully functioning innate immune system, do not fully clear a primary HSV-1 infection from their corneas (Banerjee, Biswas et al. 2004). HSK has been theorised to be an autoimmune disease based on CD4 T cell response which are regarded as the primary mediators of HSK (Niemialtowski and Rouse 1992) . One theory is that HSK occurs when the immune system cannot produce HSV specific CD8 and CD4 T lymphocytes. In a study HSK failed to develop in mice provided with a source of $CD8^+$ lymphocytes (Gangappa, Deshpande et al. 1999) indicating a roll of the adaptive immunity in keeping HSV-1 in its latent form in the trigeminal ganglia. It also seems that the severity of the disease can depend on activation of $CD4^+$ Tcells by cytokines released by HSV infection (Gangappa, Deshpande et al. 1999) and mice lacking only $CD4^+$ T cells develop less severe HSK (Lepisto, Frank et al. 2006). This coincides with findings that $CD4^+$ T cells are more abundant than $CD8^+$ Cells in the corneas with HSK (Doymaz and Rouse 1992). It seems that both $CD4^+$ and $CD8^+$ T cells mediate HSK by the same mechanism but $CD8^+$ disease is less severe (Lepisto, Frank et al. 2006). The severity of the HSK has been related to the recruitment of neutrophils to the area. It has been theorised that $CD4^+$ T cells that produce cytokines IFN-γ and IL-2 play a large role in neutrophils induction into the cornea and suppression of these cytokines has lessened the severity of the disease in animal models (Hendricks, Tumpey et al. 1992; Tang, Chen et al. 1997). IL-17 was also shown more recently to play a part in infection with it being detected in infected corneas and its suppression reduced the severity of the HSK (Suryawanshi, Veiga-Parga et al. 2011).

Treatments for HSK include topical creams and antiviral drugs such as acyclovir and trifluorothymidine (Rowe, St Leger et al. 2013). Vaccines have been tested but have do not effectively prevent reactivation or viral efficiency. A promising study showed that HSV specific $CD8^+$ T cells transferred to a mouse with latent infection seemed to establish themselves in the trigeminal ganglia. This seems to occur without replenishment from the blood of the host and seems like a great channel to explore as a vaccine towards reactivation of latent virus(Mercadal, Bouley et al. 1993).

1.5 HSV-1 and the innate immune response

HSV-1 is spread from host to host by direct contact. Herpes Simplex Keratitis is an ocular surface disease which can manifest in a person who has previously been exposed with HSV-1 at another site or can occur as a primary disease in a subject who has no history of the disease. This autoinoculation of active HSV-1 from another area of the body, commonly a cold sore, can result in HSK. The mouth is widely regarded as the main site of spread of HSV-1 in the community. HSK rarely occurs during primary ocular infections but usually presents as a reactivation of latent HSV-1 in the trigeminal ganglion with transport of the virus into the corneal stroma. Spontaneous reactivation of the virus tends to happen in humans and not in animal models. This may be due to humans being more susceptible to the conditions that cause reactivation (UV light, suppressed immune systems due to stress or fatigue, for example) which lower T cell production and therefore lower IFN-γ levels (Freeman, Sheridan et al. 2007). Another reason may be HSV's ability through a protein called ICP47 to block transport of peptides into the endoplasmic reticulum and load onto MHC class I molecules. ICP47 is one of the first proteins to be synthesised by the immediate early genes or α genes and their expression does not require previous protein synthesis. ICP47 is a cytosolic polypeptide and it prevents the MHC class I molecules from presenting antigen to $CD8^+$ Tcells and blocking their response. It achieves this by binding to the Transporter associated with antigen processing (TAP) which is essential for the loading of the MHC I cells and has a higher affinity in humans than in mice (Goldsmith, Chen et al. 1998). Active viral replication is caused when a stress stimulus reactivates the virus from its latent form and new virus returns to sites that previously contained lesions. Again neutrophils and lymphocytes are recruited into the cornea and the area undergoes inflammation and vascularisation. This ongoing periodic inflammation of the cornea over time leads to thinning, scarring and eventually a corneal transplant may be required.

Replication lasts for 5-6 days following infection with the HSV-1 virus. Normally the replicating virus doesn't reach deeper tissues such as the stroma. However, in immuno-compromised subjects the viruses commonly reaches deeper tissues and, in certain cases,

even the brain causing encephalitis and death. Replication also takes place in the trigeminal ganglion where latency is permanently established. It has been shown that humans without TLR-3, a recognition receptor found on the endosomes of dendrites and responds to the double stranded DNA of viruses, are particularly susceptible to HSV-1 infection but this hasn't been shown in relation to HSK. TLR 3 deficiency can result in impaired IFN β and IFN λ which can result in high levels of viral replication. It has been thus found that TLR3 mediated immunity is essential for protective immunity against HSV-1 (Guo et al,2011).

1.6 HSV-1 infection and adaptive immunity

HSK begins with a primary infection by HSV followed by a period where the virus remains dormant in sensory and autonomic ganglia. The virus in the ganglia is controlled by components of the innate and adaptive immune systems, with $CD8^+$ T cells playing a particularly important role (Lang and Nikolich-Zugich 2005). TLR 9 induced chemokines are suggested to help infiltrate the trigeminal ganglia with T cells and macrophages to clear the replicating virus (Lima, Zolini et al. 2010). The virus can then reactivate and although the mechanisms are not known the establishment and reactivation from latency involves the products of both the ICP0 and LAT genes. $CD8^+$ T cells have been shown to block reactivation in the TG through IFN-γ. However, IFNγ alone has been shown to block only 50% of latent HSV1 virions in trigeminal ganglion from reactivation, whereas $CD8^+$ T cells cause total blockade suggesting an additional mechanism used by $CD8^+$ T cells to block reactivation (Decman, Kinchington et al. 2005). Once the HSV-1 infection has occurred there is a release of proinflammatory cytokines to recruit neutrophils and mononuclear lymphocytes into the cornea. The eye strives to maintain corneal transparency and vision and it does this by trying to limit inflammation, immune responsiveness and neovascularisation of the normally avascular cornea (Rowe, St Leger et al. 2013).

1.7 Toll like receptor signalling in HSV-1 infection

Toll like Receptors (TLRs) are proteins that are involved in the innate immune system of vertebrates. They are known as pathogen recognition receptors that recognise molecules associated with pathogens but distinguishable from the host the host cells. When they come into contact with these pathogens they respond by sending an intracellular signal through the phosphorylation and ubiquitination to the nucleus to start producing pro inflammatory cytokines and Interferons(Akira and Takeda 2004).

HSV-1 is a large double stranded DNA virus with a genome of approximately 152kb. It belongs to the human herpes virus family. It consists of a core containing the genome, a surrounding capsid the viral matrix or tegument and an envelope. The envelope contains attachment glycoproteins, structural proteins and immune escape proteins. Most evidence suggests that the herpes virus obtains its envelope from the host cell. Six different mechanisms have been proposed for HSV recognition as seen in Figure 2. The receptors which recognise HSV include TLRs 2,3, and 9 and HSV infection stimulates the production of cytokines of which are a key focus of this project including Tumour Necrosis Factor-α(TNFα), Interleukin-6 (IL-6), Interferon α (IFNα), Interferon β (IFNβ) and Interferon γ (IFNγ). Toll like receptors recognise pathogens and through a series of intracellular signalling induced proinflammatory cytokines. TLR2 and TLR4 recognise viral proteins on the cell surface whereas TLR 3 TLR 7, TLR8 and TLR 9 are receptors for viral nucleic acids in endosomes. HSV is recognised by TLR 2, TLR 3 and TLR 9(Jin, Qin et al. 2007).

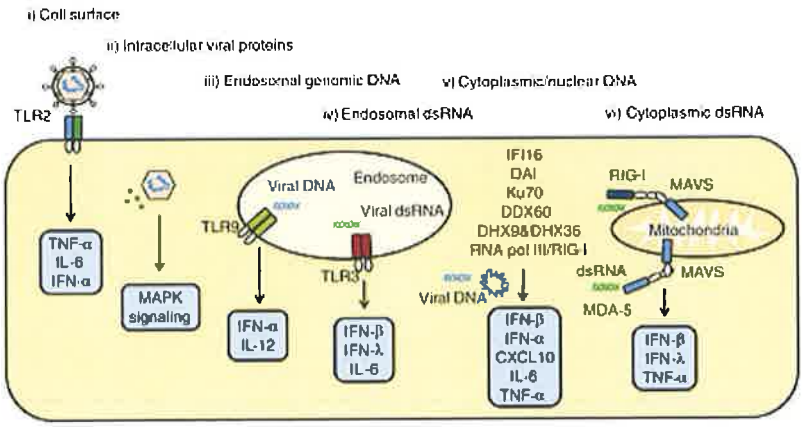


Figure 2: The various a host cell recognises HSV-1 (Melchjorsen 2012)

TLRs are part of a larger family that includes the interleukin receptors and although the two are similar in the cytoplasmic region they differ significantly in the extracellular region. Invasion of the body by pathogens triggers an immune cell response which begins once the pathogen is recognised by the TLR and triggers an inflammatory response and recruits neutrophils and lymphocytes to the site of infection. There are 10 classes of TLRs in humans each recognise a different ligand associated with a particular invading pathogen. The pathogen motifs recognised by the host cells are conserved and referred to as pathogen associated molecular patterns (PAMPs). Toll like receptors are very important for viral recognition. Certain TLRs recognise RNA produced by the viruses during replication and trigger immune responses that release Interferons and other cytokines to control the infection as well as signalling molecules to recruit other immune cells to the site of infection. Although there is conservation amongst the extracellular domains of TLRs certain ones can recognise several structurally different ligands (Akira and Takeda 2004). After ligands bind to the receptors the TLR's undergo conformational change in order to recruit downstream signalling molecules. Most of the TLRs signal through an adaptor molecule myeloid differentiation primary response protein 88(MyD88) or the TIRAP/MAL adaptor which is essential for the TLR4 MyD88 signalling pathway and TLR3 which can signal through the TRIF/TRAM pathway. All pathways result in the activation of nuclear factor (NF)-κB and activating protein -1 (AP-1). TLR 3 activates IRF3. Activation of NF- κB, IRF3 and AP-1 are all required for induction of Type 1 IFN as well as inflammatory cytokines.

In HSV-1 infected corneas a number of TLR ligands are activated. Various PAMPS including virus surface structures are recognised by the host immune system. Glycoproteins associated with the TLR 2 receptor, tegument proteins and the capsid that trigger an immune response which is not well known. TLR 2 is directly upregulated as HSV-1 activates nuclear factor-κB (NF- κB) in HEK 293 cells transfected with TLR 2. (Kurt-Jones, Belko et al. 2005). TLR 2 can recognise HSV infection but host immune systems will respond independent of TLR 2 pathways. TLR 2 knockout mice produce similar levels of TNF-α as wild types. (Mansur, Kroon et al. 2005) as well as certain cytokines and chemokines being produced independently of TLR2. The ligand for TLR2 that responds to viruses has not been identified (Takeda, Kaisho et al. 2003).

TLR 3 primarily recognises dsRNA which is produced by most viruses during their replication. The activation of the TLR 3 receptor results in the activation of NF- κB and the production of type I interferons (IFNs). TLR 3 produces cytokines dependant on the adaptor protein MyD88 but can induce NF-κB and MAP kinases independently. Polyinosine-polycytidylic acid induces TLR 3 activation in human cells.

TLR 7 is induced in macrophages upon viral infection leading to Interferon-α production. It has been demonstrated that TLR 7 and 8 recognize the single stranded RNAs found in many viruses such as human immunodeficiency, influenza A, and vesicular stomatitis virus. In cultured Human telomerase immortalised corneal epithelial cells TLR 7 is expressed after 8 hours post infection with HSV-1 (Li, Zhang et al. 2006). The natural ligands of TLR 7 still remain largely unknown but it works together in the TLR 9 subfamily which includes TLR 7, 8 and 9 to differentiate between the different nucleic acid-like structures in pathogens (Takeda, Kaisho et al. 2003).

TLR 9 is involved in the recognition of bacterial CPG DNA as well as HSV genomic DNA that is unmethylated. It mediates the expression of IFNs and a number of chemokines in dendritic cells (Sato, Linehan et al. 2006). Mice lacking TLR 9 produce less IFN-α and IL-12 but these mice can combat localised HSV infection just as well as control mice (Krug, Luker et al. 2004). In the brain TLR 9 plays an important role in restricting HSV infection.

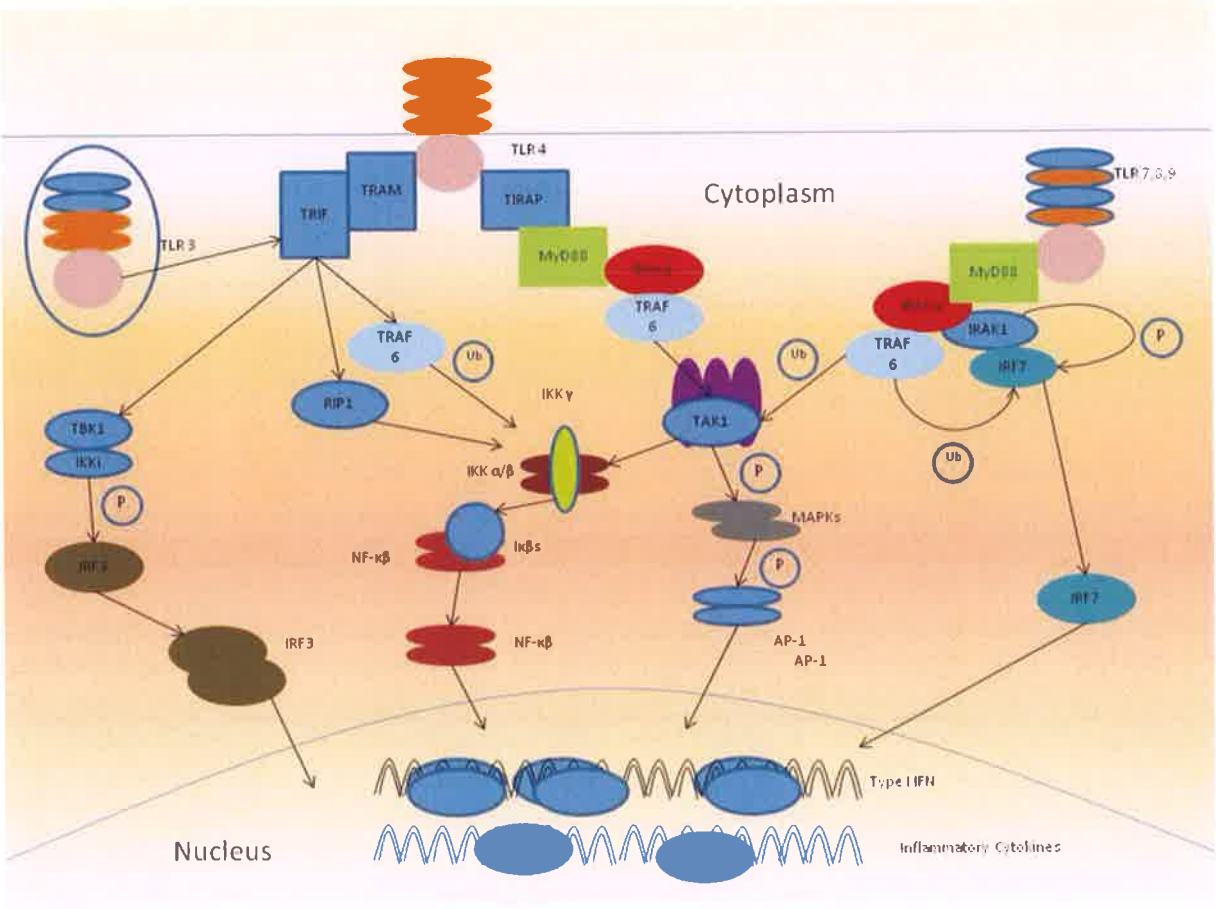


Fig 3 : Toll like receptor signalling. TLR's associated with viral infections and their respective pathways resulting in the release of inflammatory cytokines from the nucleus. (adapted from literature by author).

Type I interferons and proinflammatory cytokines are important molecules that are released and signal to attract leukocytes to the site of infection. The innate immune system is initiated by the response of Pattern Recognition Receptors (PRRs) which detect pathogen associated molecular patterns (PAMPs) which signals the presence of infection to the host

and results in the release of interferons and cytokines including Interleukins and Tumour Necrosis Factor α (TNF-α). Cells respond to a virus by activating a number of signalling pathways, including Nuclear Factor kappa B (NF-κB), IFN Regulatory Factors (IRFs) and Mitogen Activated Protein Kinase (MAPK). HSV has evolved different strategies in order to evade the production of interferons and cytokines. These include directly affecting the JAK/STAT signalling pathway and inhibition of transcription factors NF-κB and IRF3 and IRF7 signalling pathways. IFNs α & β are key to control and inhibition of HSV replication and are produced within the very first hours. These substances activate an immune response by recruiting different immune cells to the site of infection. It has been shown that mice lacking functional STAT1 are more prone to HSV encephalitis as it is an essential component of the IFNα/β pathway (Dupuis S, 2003). It is unclear whether IFNs directly act as an antiviral or regulation of the immunoregulatory system. HSV induced IFNα/β and pro-inflammatory cytokine Rantes are dependent on the functionality of the gene PKR (Malmgaard 2004, Melchjorsen 2002).

When a virus is detected the first line of defence is the activation of a series of multiple pathways NF-κB, MAPK and IRF. The JAK/STAT pathway is not directly activated but many cytokines that produced during HSV infection mediate phosphorylation of JAK and STAT proteins. HSV has several mechanisms that it uses to avoid the anti viral defences of the host cell. It uses several products to diminish IFN signalling and block the action of PKR and the (2′-5′–OAS)/RNaseL system. Also HSV can inhibit the transcription factors NF-κB and IRF3 and IRF7. It also interferes with cytokine mRNA stability and translation. IFN α/β/γ activity is heavily reliant on the Stat1 pathway this is evidenced by the fact that mice and humans deficient in STAT1 are very susceptible to HSV infections.

HSV can evade the immune response through various mechanisms that is has at its disposal. It interferes with the activity of the antiviral protein PKR which is recruited by IFNα/β. Stat 1 antiviral activity is overcome by the viral protein ICP0 and recent findings show that HSV lacking ICP0 does not suppress IRF3 and STAT1 activity(Everett, Young et al. 2008).

1.8 Innate immune evasion mechanisms of HSV.

Several countermeasures against the host IFN and proinflammatory response have been established by HSV. For instance, the IRF3 pathway is inhibited by ICP0, ICP27, and ICP34.5, through distinct mechanisms. Also, the HSV L gene products ICP34.5 and Us11 inhibit PKR activation, with Us11 exerting its action by direct binding to dsRNA, for which reason Us11 potentially also blocks activation of TLR3 and RLRs. The 2′–5′-OAS/RNAse L system is inhibited by 2′–5′ analogs. JAK/STAT signalling and the production of IFN-α/β are counteracted by a mechanism involving ICP27. Viruses lacking a functional ICP27 give higher levels of IFn-α/β in Macrophages (Melchjorsen, Siren et al. 2006). Another determinant of HSV virulence is the multifunctional protein Vhs. Recent findings have shown that vhs inhibits the JAK/STAT signalling pathway and IRF7 expression. It has been shown that vhs deficient HSV results in impaired IFN expression (Yokota, Yokosawa et al. 2004). Also HSV has been shown to upregulate the proteins suppressor of cytokine signalling (SOCS) 1-3 which has been shown to inhibit IFN signalling (Yokota, Yokosawa et al. 2004). Although HSV has many ways to evade the immune response most people who are infected with HSV do not present recurrent outbreaks of corneal ulceration.

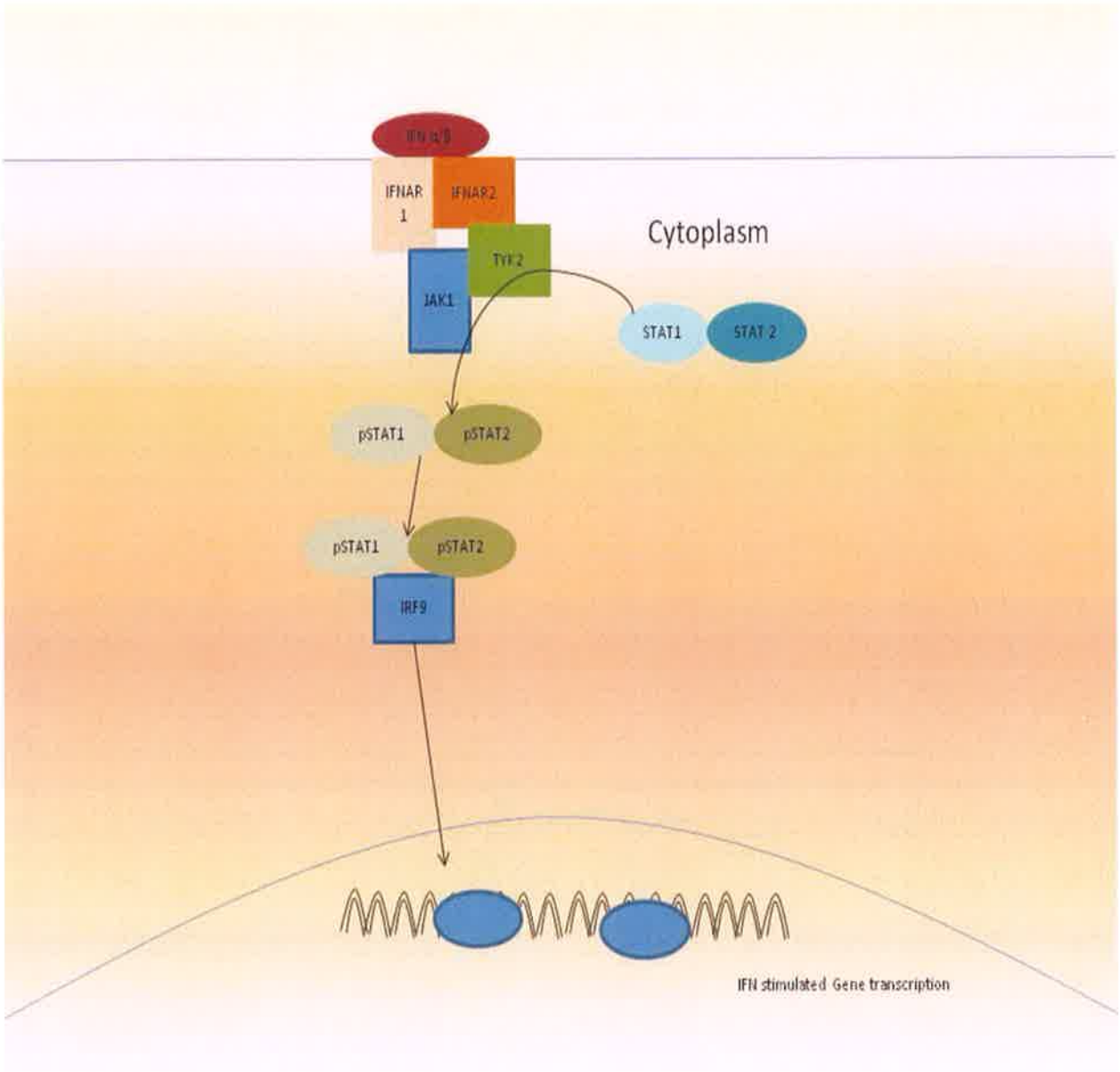


Fig 4 : Diagram of the JAK/Stat pathway induced by type I interferons (adapted from literature by author).

1.9 Aims and objectives.

During an outbreak of keratitis, the HSV virus reactivates from latency in the trigeminal ganglion due to conditions such as exposure to UV light, stress, changes in sex hormones and immune suppression. These immune stressors can all impact on T cell function which is involved in blocking the virus from reactivation (Blyth, Hill et al. 1976; Bonneau, Sheridan et al. 1991). The patients that present at the Royal Victoria Eye and Ear clinic have been diagnosed by a senior ophthalmologist with active keratitis and are therefore presenting lesions and inflammation associated with the disease. During this study, we have examined the levels of these cells in the blood of patients with active HSK caused by the HSV-1 and compared it against another blood sample from the same patient on a return visit, four weeks later, when they have been diagnosed with inactive disease this means they have undergone a prescribed treatment and they are no longer presenting any of the symptoms associated HSK upon re-examination.

The aims of this study are :

- HSV-1 infection of corneal epithelial cells results in the upregulation of various viral TLR's and a triggered immune response. But HSV-1 somehow manages to evade the Interferon immune response in particular patients by interfering with production and signalling of pathways necessary for the production of these type 1 interferons. We want to examine these immune responses in acute stage of the disease and compare them with the latent stage and also with immune responses in healthy controls.
- We aim to determine any irregularities in the immune systems of patients who suffer recurrent HSV-1 infection in the eye. The resulting HSK causes blindness in all ages and requires frequent hospital visits and may result in a high cost corneal transplant with only 66% of transplants remaining clear after 2 years (Williams, Muehlberg et al. 1995). With this in mind the need for novel approaches to management of this

disease is both important as an economic benefit as reducing patient returns to emergency rooms and outpatient hospital clinics.

- To determine any irregularities, peripheral blood mononuclear cells (PBMC's) were isolated from the patients blood. Our aim was to investigate the expression of TLRs in PBMCs and examine the production of chemokines and cytokines in patients with HSK before and after standard treatment and compare them with healthy controls
- There are treatments available but with oral acyclovir the chances of reoccurrence is reduced by only 48%. This project was established to add to the current understanding of this devastating disease on vision.

2. Materials and Methods

2.1 Patient recruitment to study and blood sampling

Patients with active Herpes Simplex Keratitis (HSK), which is characterised by the presence of a distinctive ulcer or inflammation in the cornea (clear window at the front of the eye). HSK patients typically present complaining of a sore, red, watery eye and blurring of vision. For the purpose of this study, HSK patients were examined and subsequently diagnosed by an ophthalmologist in the corneal clinic or in the Accident and Emergency Department of Royal Victoria Eye and Ear Hospital in Dublin. Upon diagnosis, patients were provided with an information sheet detailing the aim of the study. Suitable candidates that were happy to take part in the study then signed an informed consent form and were asked to provide a blood sample on the day of the diagnosis. 60ml of blood was drawn into a syringe containing 6mls sodium citrate (3.8% w/v). Then patients were commenced on standard treatment and were followed up at regular intervals, i.e. every 2-6 weeks until the infection had resolved. A second blood sample was obtained when the disease was determined to be inactive based on examination by an ophthalmologist. This study was approved by the Royal Victoria Eye & Ear Hospital (RVEEH) ethics committee.

2.2 Isolation of Peripheral Blood mononuclear Cells

Peripheral blood mononuclear cells (PBMC's) were isolated from 60ml of either healthy control or HSK patient blood. Healthy Controls were age matched +/- 5 years to the patient sample. Blood was separated into 4 x 15ml aliquots and diluted 1:1 with PBS (Biosera).This diluted blood was carefully added on top of 15ml of Ficoll Plaque Plus reagent (GE Healthcare) in 50ml tubes, being careful not to mix the solutions. Tubes were centrifuged at 300rcf for 30mins with no brake or accelerator. The white blood ring fraction from two tubes was transferred and combined into a separate 50ml tube. 1ml of plasma Serum was placed in a 1.5ml eppendorf labelled and frozen at -80°C. The volume remaining in the tube was adjusted to 50ml using PBS. The tubes were centrifuged (400rcf, 5 mins, with brake) and a pellet should form. The supernatant was poured into 2 more tubes and each pellet was resuspended in red cell lysis buffer (Sigma). Volume was again adjusted using PBS and the samples centrifuged (400rcf, 5 mins, with brake). The resulting pellets were pooled in 10ml in Roswell Park Memorial Institute (RPMI) 1640 medium containing stable glutamine, 10% (v/v) foetal calf serum (FCS) and 100µg/mL penicillin/streptomycin (P/S). FCS was heat inactivated in 56°C for 30 min. to inactive complement and aliquoted for storage at -20°C. Modified medium was stored at 4°C. Cell counting and viability was determined using the dye Trypan blue, which is taken up by non-viable cells but not from healthy cells. The cells were then counted using a haemocytometer and a bright light microscope. PBMCs were seeded into 96 well plate at 2.5 x 10^5/ml and stimulated for 24 hrs' 6 well plates at 1x 10^6/ml and stimulated for 4 and 8 hours 24 well plates at 1x10^6cells/ml and stimulated at 0.5, 1, 3, 6 and 24 hours and incubated in a 37°C incubator with 5% CO_2 (Model 381-Thermo Electron Corporation, OH, USA). They were then stimulated with polyinosinic:polycytidylic acid (poly I:C) (20µg/ml), lipopolysaccharide (LPS) (100ng/ml), and imiquimod (10µg/ml).

2.3 ELISA

96 well high binding plates (Nunc) were coated with capture antibody overnight at a concentration specified in the manufacturers datasheet. Plates were washed 3 times with ELISA wash buffer (PBS 0.05% Tween) before blocking for 1 hour with Bovine Serum Albumin (1%). Standards were made up according to the manufacturers datasheet ranging from 2000 – 0 pg/ml. Standards and sample were added to wells (50ul) and shaken (1hr, RT, 900rpm). Plates were washed 3 times in wash buffer. Detection antibody (50ul) was added at a concentration according to the manufacturers datasheet and plates were shaken (1hr,RT, 900rpm) before washing 3 times with wash buffer. HRP was added (50ul, 1:200) to the plate and the plate was shaken in the dark (20mins,RT,900rpm). The plate was washed 3 times in wash buffer. TMB (50ul) was added and the plate was shaken in the dark (20mins, RT, 900rpm). H_2SO_4(50ul) was added to stop the colour change. The plate was read on a WALLAC plate reader(450nm, 0.1s). ELISA was used for the analysis of TNFα, IL-6, Rantes and IFN-γ.

2.4 FACS

Flow Cytometry is used for biomarker detection using a laser and fluorescence detectors. The laser at a particular wavelength is aimed at sheath fluid containing the cells of interest and each suspended particle passing through the laser beam scatters the ray depending on size and fluorescent tags on the cells can be detected by various emission. Total cells were counted but the cells of interest were gated using the FACSdiva software accompanying the machine. The Flow cytometer used in this study was a BD FACS Canto II with the ability to detect 6 fluorescent tags listed in table (Fig 2.4.1).

PBMCS were isolated as explained above and 1ml was transferred at a concentration of $1x10^6$/ml to 5 FACS tubes. Antibody mixes were prepared in two tubes one for T cells and NK cells and the second mix for B cells and macrophages (figure 1.1). Isotype controls were prepared for each fluorochrome to adjust for background. 7 tubes were set up for compensation. Negative beads were added to one with negative and positive beads and one of each flurochrome were added to the remaining 6. Compensation was calculated on the FACS and applied to the samples. The cells were centrifuged (5mins, 1600rpm) and then blocked on ice using human IgG (1hr, 200µg/ml, Sigma). The cells were then added to the antibody mix and incubated on ice for 1hr. The cells were washed of excess antibody with FACS wash buffer 2% FCS in PBS (2mls) which was added to each tube and the cells were centrifuged (5mins, 1600rpm) and resuspended in FACS wash buffer (200ul)

For our purposes two antibody mixes were made up. One was to identify specific activation markers on T cells and Natural Killer cells (NK) and the second mix was to identify B cells and Macrophages. CD3 was used as an immunohistochemical marker for T cells as it is expressed at all stages of T cell development. The two types of T cells $CD8^+$ and $CD4^+$ were distinguished using individual antibodies labelled with different fluorophores. Natural Killer cells were isolated using a labelled CD56 antibody, CD56 being a glycoprotein expressed on the surface of NK cells and not T cells. For the T cell/NK cell mix we investigated the changes in CD69 and CD 25 expression levels on each of the cells. CD25 is the alpha chain of the IL-2 receptors and is present on activated T cells. CD69 appears to be the earliest cell

surface glycoprotein acquires during activation and functions as a signal transmitting receptor in T cells and NK cells.

	Tcells/NK cells		Bcells/Macrophages	
FITC	CD8	IgG_1	CD80	IgG_1
PE	CD69	IgG_1	CD86	IgG_1
PE-Cy5	CD4	IgG_1	CD19	IgG_1
PE-Cy7	CD56	IgG_1	Class I	IgG_1
APC	CD25	IgG_1	Class II	IgG_{2b}
APC-Cy7	CD3	IgG_1	CD14	IgG_1

Fig 2.4.1 : Antibody mixes for FACS analysis, each mix was incubated with human PBMCs and analysed using BD FACS Canto II. All cells were counted and cells of interest were isolated using the FACSdiva software package.

2.5 SDS PAGE/Western blotting

Human peripheral blood mononucleated cells were isolated as described above and seeded on a 24 well plate at $1x10^6$cells/ml. The cells were incubated at 37°C and stimulated with ligands polyinosinic:polycytidylic acid (poly I:C) (20µg/ml), lipopolysaccharide (LPS) (100ng/ml), imiquimod (10µg/ml) and CpG oligodeoxynucleotide A (CPGa)(3µM) at time periods 0.5, 1, 3, 6 and 24 hours. After stimulation, cells were lysed in 200µL of 1xSDS sample buffer [50mM Tris-HCl, pH 6.8, 2% SDS, 0.1% Bromophenol blue, 10% glycerol]. Immediately prior to use, 50µL of dithiothreitol (DTT) was added per mL of sample buffer. Samples were then sonicated for 3x5 sec at 40% amplitude and boiled at 95°C for 5 min. to reduce the DTT.

Using the Atto Gel Electrophoresis system, 8% resolving gels [4mL Protogel (30% acrylamide:bisacrylamide), 6.9 mL water, 3.8mL 1.5M Tris-HCl pH8.8, 0.15mL 10% SDS, 0.15mL 10% ammonium persulfate (APS) and 6µL tetramethylethylenediamine (TEMED)] and stacking gels [1mL Protogel, 4.1mL water, 0.75mL 1M Tris-HCl pH 6.8, 60µL 10% SDS, 60µL 10% APS, 6µL TEMED] were prepared. 6µL of pre-stained protein markers were run alongside 20µL samples as molecular weight standards. The samples were resolved through the gel for 1 hour at a constant current of 35mA per gel using running buffer [25mM Tris, 192mM glycine, 0.1% SDS]. Using a Trans-Blot Electrophoretic Cell (Bio-Rad), resolved proteins were transferred onto a 0.45µm Immobilon-P Transfer Membrane (TM) polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was then soaked in methanol for 10 sec. and rinsed in transfer buffer [25mM Tris, 192mM glycine, 20% methanol]. The gel and membrane were placed in a cassette, sandwiched between filter paper and fibre pads while soaked in transfer buffer. The cassettes were then placed in the chamber, which was filled with transfer buffer and a constant current of 200mA was applied for 1 hour. To block non-specific binding the membranes were then transferred 5% milk in Tris buffered saline (TBS)-0.1% (v/v) Tween for 1hr at room temperature. The membranes were then transferred to primary antibody overnight, certain dilutions depending on the antibody (Table I) overnight at 4°C. The membrane was then washed in 1% (v/v) TBS-Tween for 3 x 10min. and incubated in the appropriate concentration of horse radish peroxidise (HRP)-linked secondary antibody for 1 hour at RT (Table I). Subsequent washes were done

for 3 x 5min in 1% (v/v) TBS-Tween. Blots were then developed by enhanced chemiluminescence (ECL) according to the manufacturer's instructions. Densitometry analysis was performed on the blots and

Antibody	≈kDa	Buffer Used	1° Antibody Dilution	2° Antibody Type/Dilution	Storage
STAT1	91/86	5% milk	1:5000 (1µL)	Rabbit/1:10000 (0.5µL)	4°C
IRF-3	57	5% milk	1:1000 (5µL)	Rabbit/1:10000 (0.5µL)	-20°C
b-Actin	44	5% milk	1:1000 (5 µL)	Rabbit/1:10000 (0.5µL)	4°C

Rabbit polyclonal antibody against STAT1 p84/p91 (M-22) and b-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibody IRF-3 was purchased from Active Motif (Carlsbad, CA).

2.6 Meso scale discovery Multi spot assay system

Calibrator stock was prepared by transferring 10µl of the human proinflammatory 7-plex calibrator blend to 990µl of diluents 2. This calibrator stock (50µl) was transferred to diluents 2 (150µl) to make the highest calibration point (Std -1). This dilution (1:4) was repeated 6 times to make 7 standards in total. The 8th calibration standard was diluent 2 neat. Detection antibody blend (60µl) was diluted in diluents 3 (2.94ml) to make a 1x detection antibody solution. 4X read Buffer T (10mls) was added to deionised water (10mls) to make 2X read buffer. Diluent 2 (25µl) was dispensed into each well. The plates were sealed and placed on a shaker for (30mins, RT, 1000rpm). Calibrator standards and samples (25µl) were dispensed into wells and the plate was sealed and placed on a shaker (120mins, RT, 1000rpm). The plate was washed with PBS-T (x3). 1X detection antibody (25µl) was dispensed into each well. The plate was sealed and placed on a shaker (120mins, RT, 1000rpm). The plate was washed with PBS-T (x3). 2X read buffer T (150µl) was added to each well. Plate was read immediately on the SECTOR imager.

2.7 Stimulations:

An aliquot of cells were stimulated with the following ligands polyinosinic:polycytidylic acid (poly I:C) (20µg/ml), lipopolysaccharide (LPS) (100ng/ml), imiquimod (10µg/ml) and CpG oligodeoxynucleotide A (CPGa)(3µM). These were to artificially stimulate TLR 3, TLR 4, TLR 7 and TLR 9 respectively.

2.7 Statistics :

All statistics were done using a non parametric or Mann-Whitney t-test. All significant results were reported using a * grading system which is explained below. Any results which showed observable trends approaching significance the p value was reported on the graph. Graphpad Prism 5 software package was used to perform all the statistical analysis.

Pvalue	Wording	Summary
< 0.001	Extremely significant	***
0.001 to 0.01	Very significant	**
0.01 to 0.05	Significant	*
>0.05	Not significant	ns

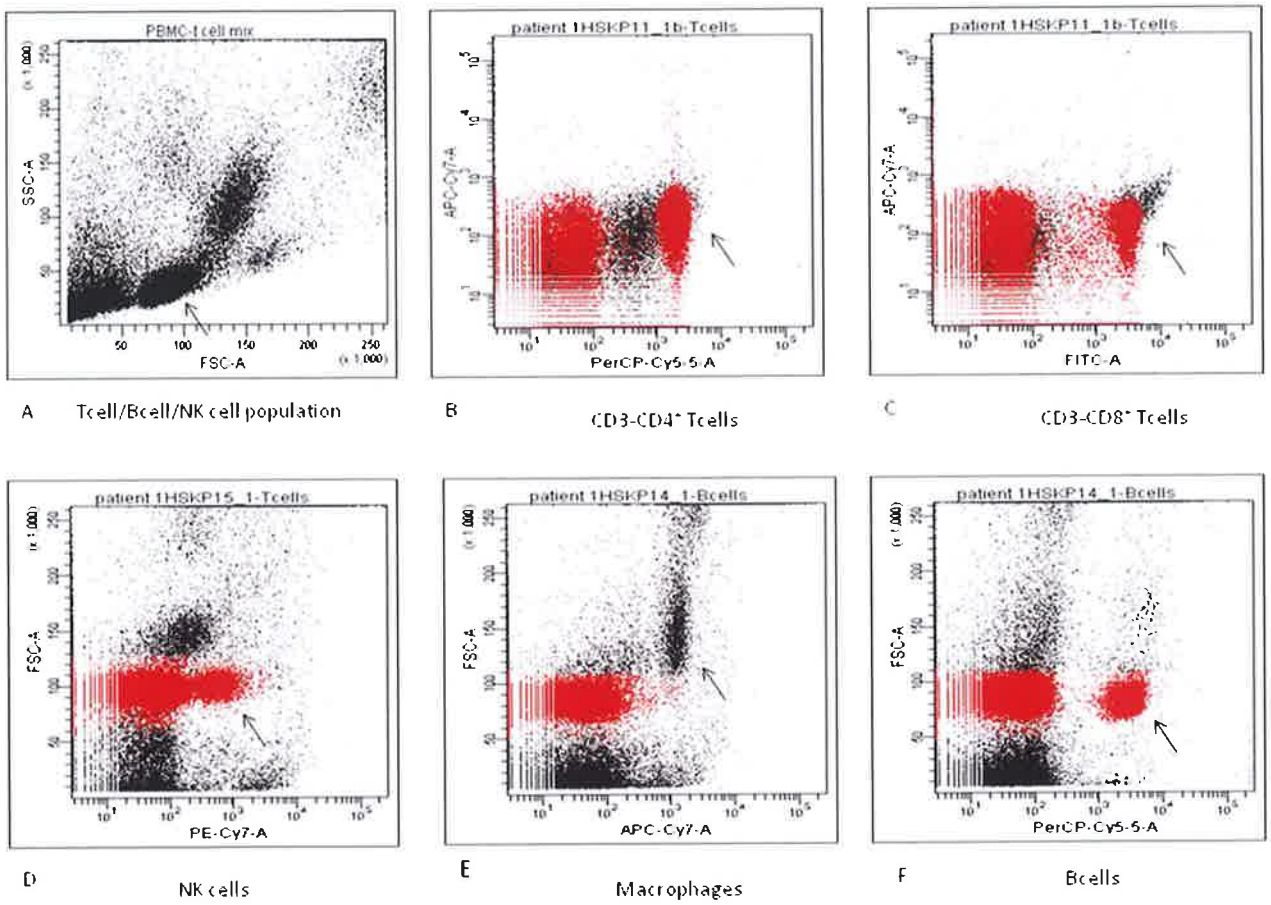
Fig 2.7.1: Legend of comparison of ELISA dot plots

3. Results

3.1 Comparative analysis of activation status of T cells, B cells and macrophages derived from active and inactive HSK patients by flow cytometry.

In order to investigate the activation status of peripheral blood immune cell populations, purified PBMCs were analysed by flow cytometry. Two antibody mixes were made up which are listed in the results section (fig 2.4.1). One was to identify specific activation markers on T cells and NK and the second mix was to identify B cells and macrophages. CD3 is used as an immunohistochemical marker for T cells as it is expressed at all stages of T cell development. The two types of T cells $CD8^+$ and $CD4^+$ are distinguished using individual antibodies labelled with different fluorophores. Natural Killer cells were detected using a labelled CD56 antibody. CD56 is a glycoprotein expressed on the surface of NK cells and not T cells. For the T cell/NK cell mix changes in CD25 and CD69 expression levels were assessed on each of the cells. CD25 is the alpha chain of the IL-2 receptors and is present on activated T cells. CD69 appears to be the earliest cell surface glycoprotein acquires during activation and functions as a signal transmitting receptor in T cells and Natural Killer cells. A representative analysis of immune cell populations is shown in figure 3.1.1

To recognise B cells the patient PBMC's were incubated with CD19 which is found on the surface of B cells. CD14 was used to distinguish macrophages. It is mainly expressed by macrophages but also can be expressed by neutrophils. Activation markers were used to compare basal levels of active disease in patients versus inactive. CD80 is a protein found on activated macrophages and Bcells. CD86 is also found in activated Bcells and macrophages and works in tandem with CD80 to prime Tcells. The major histocompatibility complex (MHC) class I and II were analysed using specific antibodies. MHC molecules are mostly found on antigen presenting cells like B cells and macrophages. Class I and II are structurally different and present to different cells. A representative analysis of B cell populations and activation marker status is shown in figure 3.1.2.



A Tcell/Bcell/NK cell population

B CD3-CD4$^+$ Tcells

C CD3-CD8$^+$ Tcells

D NK cells

E Macrophages

F Bcells

Fig 3.1.1: Sample of populations labelled with primary antibodies that were directly fluorescently labelled. Each population is marked with a cell surface molecule unique to that type of cell. A: Shows all cells counted with Forward scatter plotted against Side scatter, this is the gated region. The T cells are shown in a dense population. B: CD3$^+$-CD4$^+$ T cells which are labelled with APC-Cy7 and PerCP-Cy5. C: CD3$^+$-CD8$^+$ T cells which are labelled with APC-Cy7 and FITC. D: NK-cells labelled with CD56-PE-Cy7. E: Shows macrophages labelled with CD14-APC-Cy7. F: Bcells labelled with CD19-PerCPCy5. The software used was FACSdiva which accompanies the BD FACS Canto II.

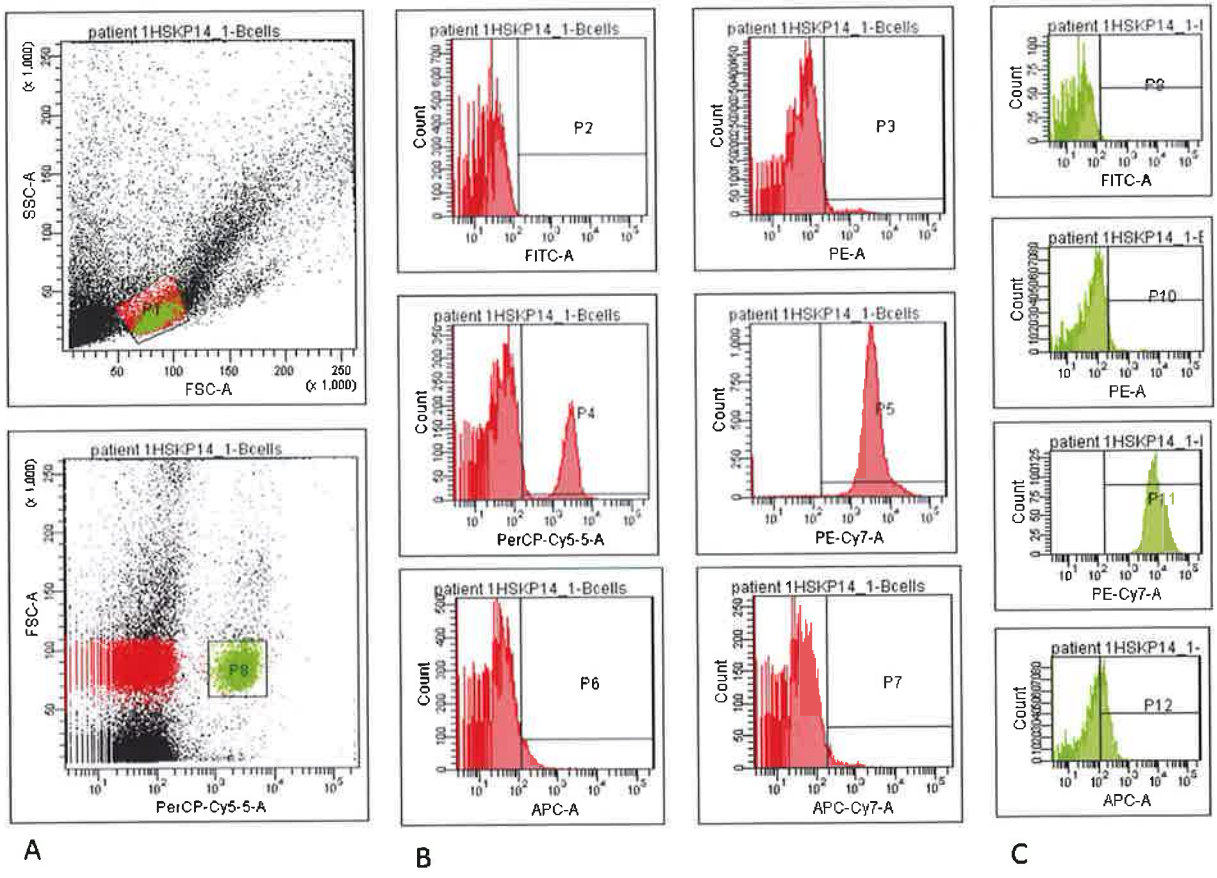
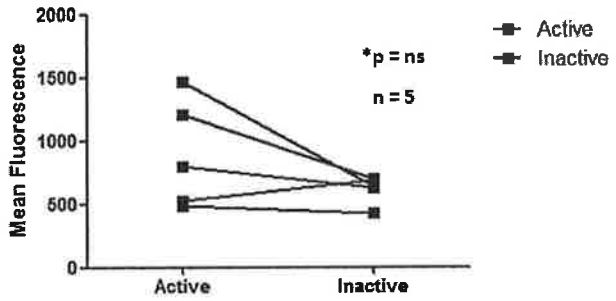


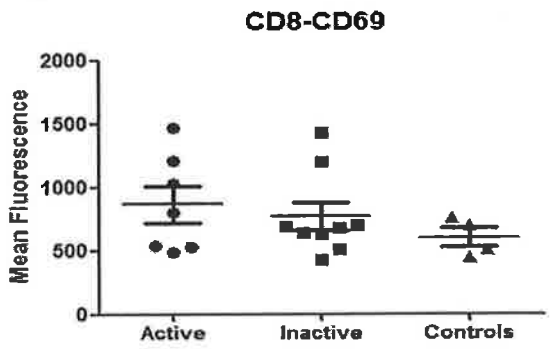
Fig 3.1.2: Example of the analysis of B cells carried out on the FACSdiva software. A: Scatter plot of total cells counted and scatter plot of B cell population isolated by tagging cells with CD19-PerCP-Cy5 tag. B: Histograms plotting cell counts against each fluorescently labelled antibody in the mix. Each graph show if there was a shift from the isotype control based on a marker P2-P7. C: Histograms showing the level of fluorescence on a specifically gated population, this is gated on the B cell population and showing. CD80-FITC, CD86-PE, MHC-Class I – PE-Cy7, MHC Class II – APC.

When $CD8^+$ T cells were analysed, assessing CD69 expression on individual patients, a trend towards a decrease in CD69 levels (as evidenced by MFI) was observed from active to inactive patients (Figure 3.1.3a). However, a Mann-Whitney t-test comparison shows these changes as not being significant ($p>0.05$) fig 3.1.3c, due to the large spread of MFI observed in the active patient sample set. This would suggest a difference in expression of CD69 in $CD8^+$ T cells in active patients, probably due to CD8+ T cell activation with active viral infection in this patient sample. Mean fluorescence for total active and inactive patients shows no observable difference which is confirmed with a Mann-Whitney t-test as not being significant ($p>0.05$) fig 3.1.3c. CD69 levels in inactive patients are mostly comparable to those seen in healthy control samples, indicating the immune system of inactive patients return to relative normality post-treatment.

A



B



C

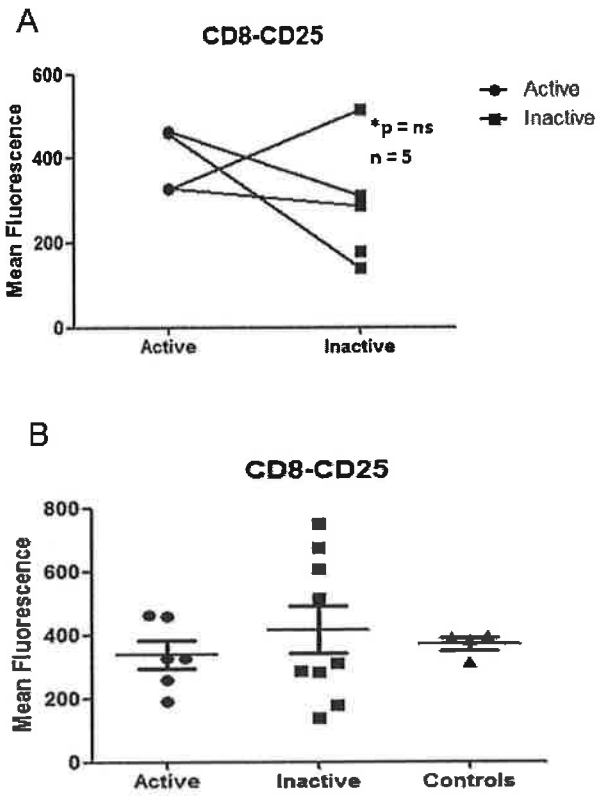
Table Analyzed CD8 - CD69	Active	Inactive
Number of values	7	9
Minimum	487	428
25% Percentile	526	566.5
Median	795	679
75% Percentile	1206	947.5
Maximum	1467	1429

Table Analyzed CD8 - CD69	Active	Inactive
Number of values	7	9
Mean	863.6	765.6
Std. Deviation	382.1	328.8
Std. Error	144.4	109.6

CD8 - CD69	Active vs Inactive
Mann Whitney test	
P value	0.6806
P value summary	ns

Fig 3.1.3: A: $CD8^+$ T cells CD69 levels of mean fluorescence from patients with active HSK and after treatment. B: Comparison of Mean fluorescence data for all active and inactive patients and healthy controls. C: Statistical information for Graph B including means and median and a Mann-Whitney test to compare data.

When CD25 expression on CD8+ T cells was assessed, a similar trend towards a decrease was observed from active to inactive patients in all but one sample (Figure 3.1.4a). A t-test comparison shows these changes as not being significant ($p>0.05$), However, when we compare all active and inactive patients (figure 3.14b) we see a greater spread in the inactive samples suggesting that CD25 does not play a role as a marker for activation for this disease as the mean fluorescence shows a lower trend in the active samples. A comparison of these differences were measured using a non-parametric Mann-Whitney t-test and found to be not significant ($p>0.05$) fig 3.1.4c.



C

Table Analyzed CD8 - CD25	Active	Inactive
Number of values	6	9
Minimum	192	140
25% Percentile	241.5	229.5
Median	327.5	311
75% Percentile	460	640.5
Maximum	463	751

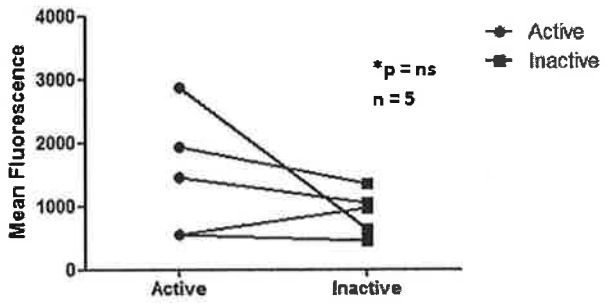
Table Analyzed CD8 - CD25	Active	Inactive
Number of values	6	9
Mean	337.8	416.1
Std. Deviation	107.9	224.7
Std. Error	44.05	74.91

CD8 - CD25	Active vs Inactive
Mann Whitney test	
P value	0.7756
P value summary	ns

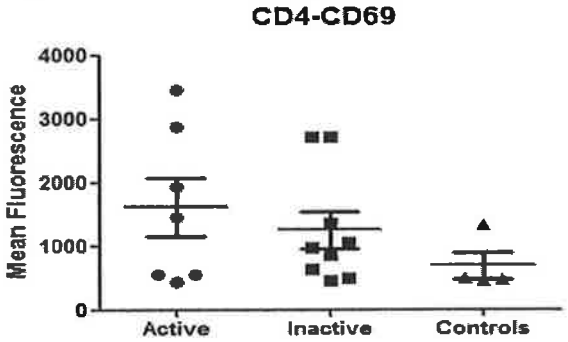
Fig 3.1.4: A: CD8$^+$ T cells CD25 levels of mean fluorescence from patients with active HSK and after treatment. B: Comparison of Mean fluorescence data for all active and inactive patients and healthy controls. C: Statistical information for Graph B including means and median and a Mann-Whitney test to compare data.

When $CD4^+$ T cells were analysed, it can be seen that there was a general trend towards increased CD69 expression on active CD4+ T cells. A Mann-Whitney t-test comparison shows these changes as not being significant ($p>0.05$) figure 3.1.5a.When we compare all active and inactive patients we see a trend towards greater spread in the active samples. These differences when examined statistically were not significant ($p>0.05$) fig 3.1.5c. CD69 levels in the healthy controls follow the trend of the inactive patient samples, indicating that infection has subsided in the inactive patients. When CD25 was analysed on CD4+ cells no significant changes were observed between active and controls (fig 3.1.6).

A



B



C

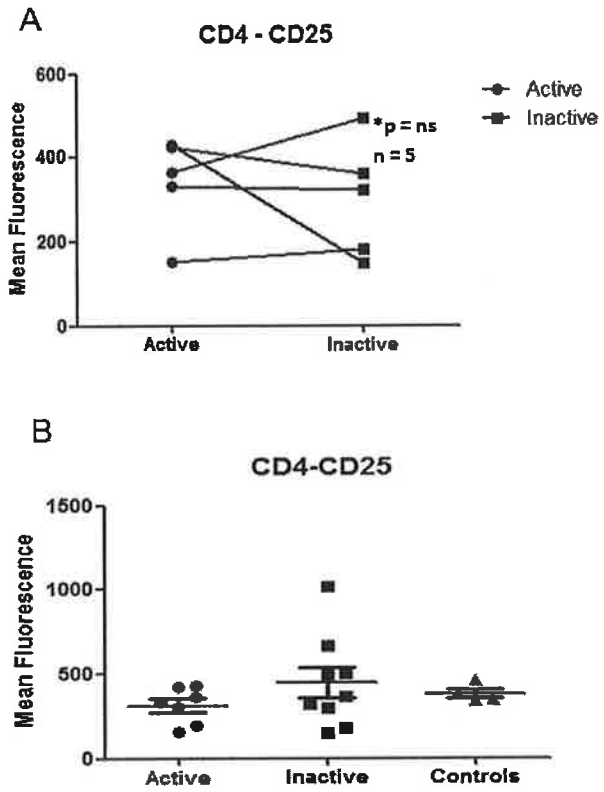
Table Analyzed CD4 - CD69	Active	Inactive
Number of values	7	9
Minimum	440	445
25% Percentile	555	564
Median	1458	966
75% Percentile	2881	2037
Maximum	3454	2713

Table Analyzed CD4 - CD69	Active	Inactive
Number of values	7	9
Mean	1612	1249
Std. Deviation	1207	877.6
Std. Error	456.2	292.5

CD4 - CD69	Active vs Inactive
Mann Whitney test	
P value	0.6806
P value summary	ns

Fig 3.1.5: A: $CD4^+$ T cells CD69 levels of mean fluorescence from patients with active HSK and after treatment. B: Comparison of Mean fluorescence data for all active and inactive patients and

healthy controls. C: Statistical information for Graph B including means and median and a Mann-Whitney test to compare data.



C

Table Analyzed CD4 - CD25	Active	Inactive
Number of values	7	9
Minimum	152	150
25% Percentile	194	240.5
Median	333	363
75% Percentile	425	582.5
Maximum	431	1021

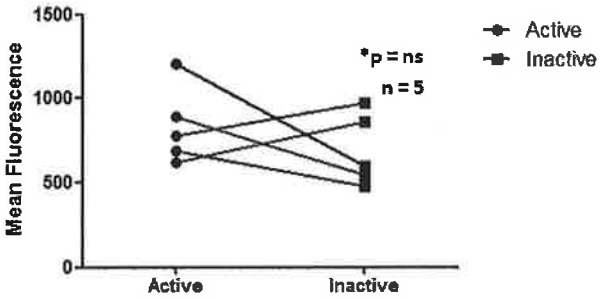
Table Analyzed CD4 - CD25	Active	Inactive
Number of values	7	9
Mean	314.9	444.2
Std. Deviation	107.9	270.7
Std. Error	40.78	90.22

CD4 - CD25	Active vs Inactive
Mann Whitney test	
P value	0.6806
P value summary	ns

Fig 3.1.6: A: CD4$^+$ T cells CD25 levels of mean fluorescence from patients with active HSK and after treatment. B: Comparison of Mean fluorescence data for all active and inactive patients and healthy controls. C: Statistical information for Graph B including means and median and a Mann-Whitney test to compare data.

Analysis of NK cells in HSK patients show no clear pattern for CD69 activation with 3 patients showing decreases and 2 showing an increase (Figure 3.1.7a) and almost no effect on CD25 expression (Figure 3.1.8). A Mann-Whitney comparison shows these changes as not being significant ($p>0.05$). Interestingly, for both CD69 (Figure 3.1.7b) and CD25 (Figure 3.1.8b) levels in the healthy controls show a smaller spread. This may indicate that CD69 levels in NK cells of HSK patients are at different states of activation compared to the general healthy population.

A



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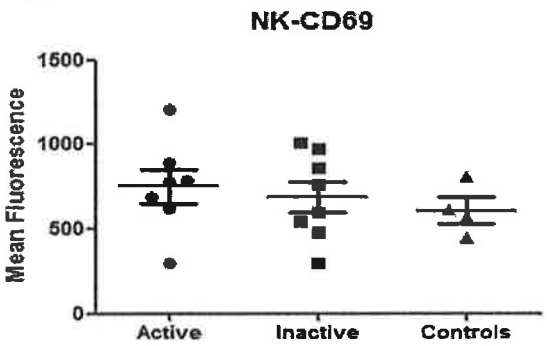


C

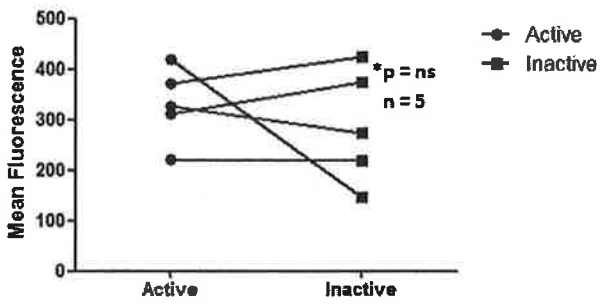
Table Analyzed NK-CD69	Active	Inactive
Number of values	6	8
Minimum	384	300
25% Percentile	416.3	524
Median	614.5	683
75% Percentile	1018	757.8
Maximum	1306	824

Table Analyzed NK-CD69	Active	Inactive
Number of values	6	8
Mean	711.3	632.4
Std. Deviation	350.2	171.6
Std. Error	143	60.67

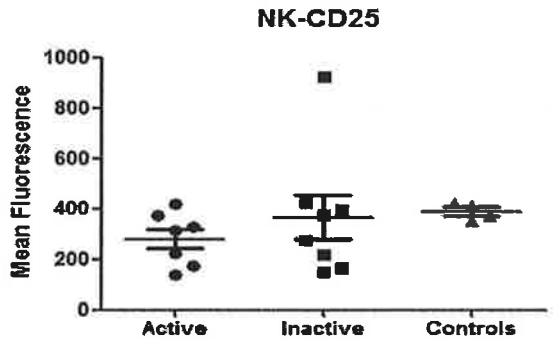
NK-CD69	Active vs Inactive
Mann Whitney test	
P value	0.9497
P value summary	ns

Fig 3.1.7: A: NK Cells CD69 levels of mean fluorescence from patients with active HSK and after treatment. B: Comparison of Mean fluorescence data for all active and inactive patients and healthy controls. C: Statistical information for Graph B including means and median and a Mann-Whitney test to compare data.

A



B



C

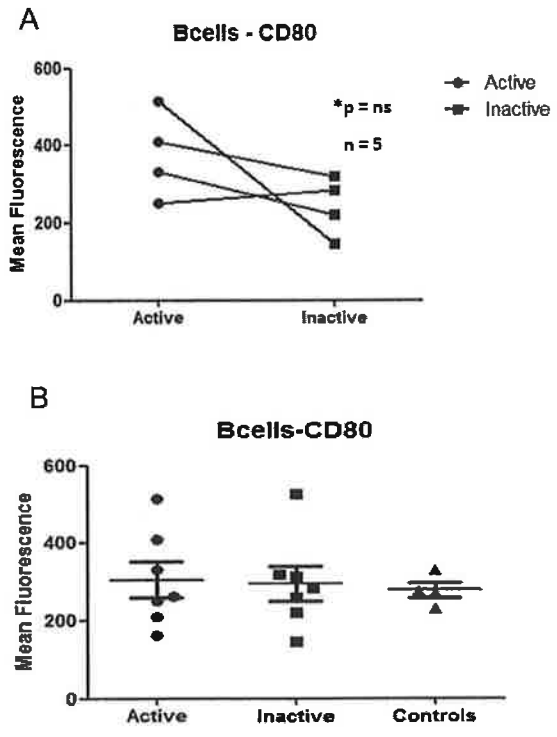
Table Analyzed NK-CD25	Active	Inactive
Number of values	5	8
Minimum	181	141
25% Percentile	226.5	190.8
Median	344	311.5
75% Percentile	453.5	522
Maximum	482	598

Table Analyzed NK-CD25	Active	Inactive
Number of values	5	8
Mean	340.8	346.9
Std. Deviation	119.7	170.9
Std. Error	53.53	60.41

NK-CD25	Active vs Inactive
Mann Whitney test	
P value	0.9433
P value summary	ns

Fig 3.1.8: A: NK Cells CD25 levels of mean fluorescence from patients with active HSK and after treatment. B: Comparison of Mean fluorescence data for all active and inactive patients and healthy controls. C: Statistical information for Graph B including means and median and a Mann-Whitney test to compare data.

When B cells were analysed for CD80 activation they showed a trend towards a decrease from active HSK to inactive state across all patients, although again these results were not found to be statistically significant (Figure 3.1.9a). When we compare all active and inactive patients we see an indication of higher values in the active patients meaning the CD80 does activate to some extent in active disease CD80 levels in the healthy controls show a similar level to HSK patients in their inactive state but more tightly grouped indicating that in inactive treated patients the levels are similar to healthy controls (figure 3.1.9b). .When B cells were analysed for CD86 activation they show no change from active HSK to inactive state across all patients or when compared with controls (Figure 3.1.10), indicating that CD86 may not be upregulated in HSK. Analysis of MHC class I and II expression on the B cells (figure 3.1.11 and 3.1.12 respectively) demonstrates little if any changes observed in expression between active and inactive patients, although their levels of these molecules was reduced compared to healthy controls. These changes were not statistically significant for Class I (3.1.11c) or Class II (fig 3.1.12c)



C

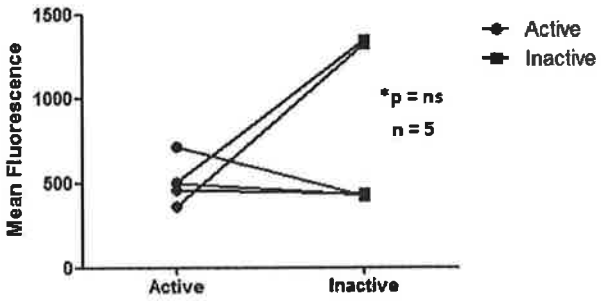
Table Analyzed Bcells-CD80	Active	Inactive
Number of values	7	7
Minimum	164	146
25% Percentile	211	221
Median	262	283
75% Percentile	409	319
Maximum	515	526

Table Analyzed Bcells-CD80	Active	Inactive
Number of values	7	7
Mean	306.4	295.6
Std. Deviation	121.8	117.9
Std. Error	46.03	44.55

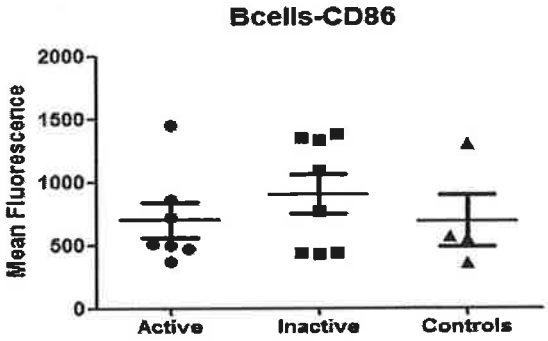
Bcells-CD80	Active vs Inactive
Mann Whitney test	
P value	1.0000
P value summary	ns

Fig 3.1.9: A: B cells CD80 levels of mean fluorescence from patients with active HSK and after treatment. B: Comparison of Mean fluorescence data for all active and inactive patients and healthy controls. C: Statistical information for Graph B including means and median and a Mann-Whitney test to compare data.

A



B



C

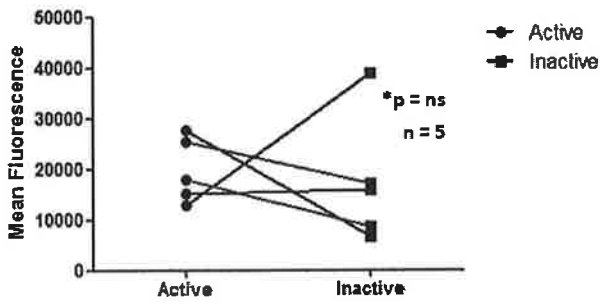
Table Analyzed Bcells-CD86	Active	Inactive
Number of values	7	8
Minimum	366	422
25% Percentile	465	433.3
Median	511	925.5
75% Percentile	855	1342
Maximum	1451	1377

Table Analyzed Bcells-CD86	Active	Inactive
Number of values	7	8
Mean	695.3	898.8
Std. Deviation	372.3	434.1
Std. Error	140.7	153.5

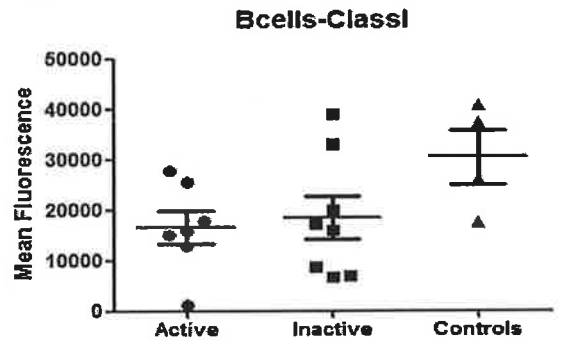
Bcells-CD86	Active vs Inactive
Mann Whitney test	
P value	0.6943
P value summary	ns

Fig 3.1.10: A: B cells CD86 levels of mean fluorescence from patients with active HSK and after treatment. B: Comparison of Mean fluorescence data for all active and inactive patients and healthy controls. C: Statistical information for Graph B including means and median and a Mann-Whitney test to compare data.

A



B



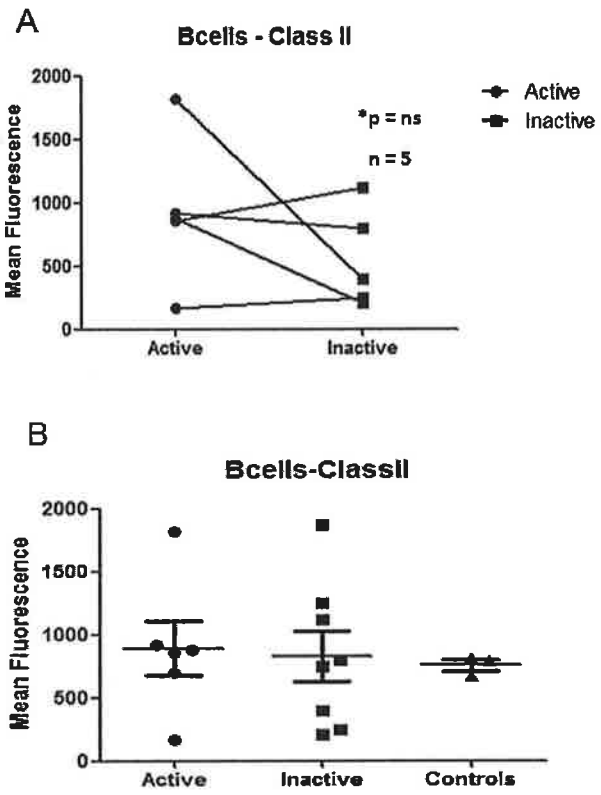
C

Table Analyzed Bcells-Class I	Active	Inactive
Number of values	7	8
Minimum	1258	6607
25% Percentile	12914	7270
Median	16041	16581
75% Percentile	25462	29600
Maximum	27649	39017

Table Analyzed Bcells-Class I	Active	Inactive
Number of values	7	8
Mean	16634	18368
Std. Deviation	8699	12024
Std. Error	3288	4251

Bcells-Class I	Active vs Inactive
Mann Whitney test	
P value	0.9551
P value summary	ns

Fig 3.1.11: A: B cells MHC Class I levels of mean fluorescence from patients with active HSK and after treatment. B: Comparison of Mean fluorescence data for all active and inactive patients and healthy controls. C: Statistical information for Graph B including means and median and a Mann-Whitney test to compare data.



C

Table Analyzed Bcells-Class II	Active	Inactive
Number of values	7	8
Minimum	169	206
25% Percentile	194	283
Median	859	770
75% Percentile	917	1212
Maximum	1817	1865

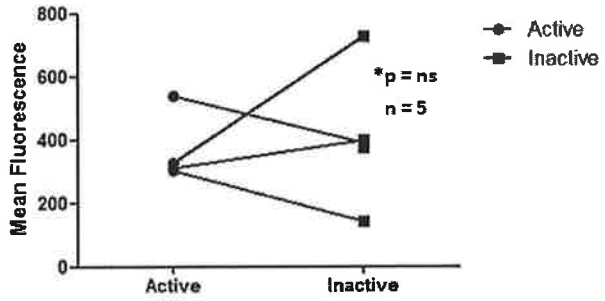
Table Analyzed Bcells-Class II	Active	Inactive
Number of values	7	8
Mean	790.1	826.8
Std. Deviation	552.5	566.9
Std. Error	208.8	200.4

Bcells-Class II Active vs Inactive	
Mann Whitney test	
P value	0.7789
P value summary	ns

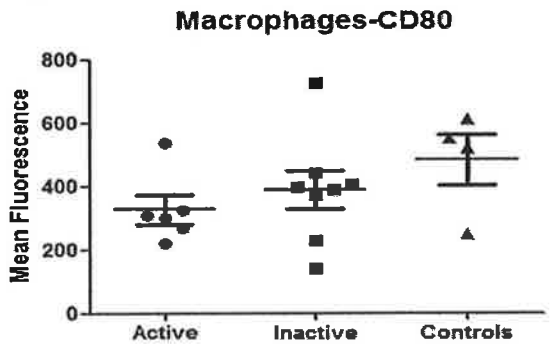
Fig 3.1.12: A: B cells MHC Class II levels of mean fluorescence from patients with active HSK and after treatment. B: Comparison of Mean fluorescence data for all active and inactive patients and healthy controls. C: Statistical information for Graph B including means and median and a Mann-Whitney test to compare data.

When analysing macrophages activation markers, no observable change in CD80 expression was observed (Figure 3.1.13), although a trend towards a decrease in active HSK was noted (p=0.345). When macrophages were analysed for CD86 activation (figure 3.1.14), a general trend towards decreased CD86 expression in activated HSK was observed which the addition of more data may become significant (p=0.1419). MHC class I expression was identical across all populations (figure 3.1.15) while class II levels were decreased in active HSK patients compared to inactive or controls (Figure 3.1.16). Again this observation seems to have a trend which may become significant with additional patient data (p=0.1419). These results may indicate a reduced activation of Macrophages in HSK patients compared to normal expected immune responses.

A



B



C

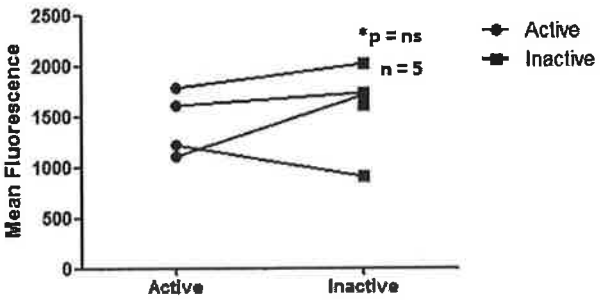
Table Analyzed Macrophages-CD80	Active	Inactive
Number of values	6	8
Minimum	224	143
25% Percentile	259.3	267.3
Median	306	394.5
75% Percentile	380.8	434.8
Maximum	539	727

Table Analyzed Macrophages-CD80	Active	Inactive
Number of values	6	8
Mean	329	389.4
Std. Deviation	109.2	170.3
Std. Error	44.56	60.22

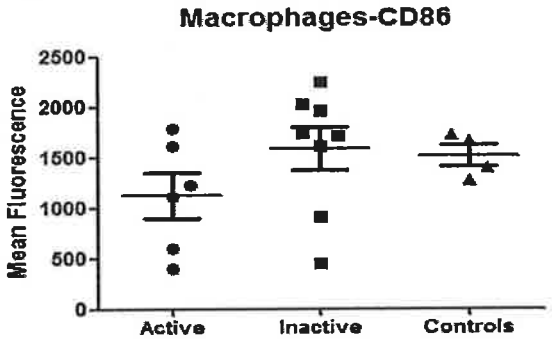
Macrophages-CD80	Active vs Inactive
Mann Whitney test	
P value	0.3450
P value summary	ns

Fig 3.1.13: A: Macrophages CD86 levels of mean fluorescence from patients with active HSK and after treatment. B: Comparison of Mean fluorescence data for all active and inactive patients and healthy controls. C: Statistical information for Graph B including means and median and a Mann-Whitney test to compare data.

A



B



C

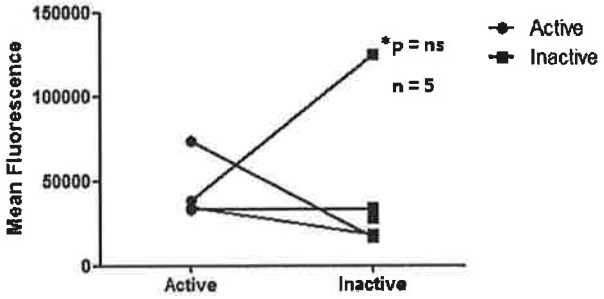
Table Analyzed Macrophages-CD86	Active	Inactive
Number of values	6	8
Minimum	396	442
25% Percentile	550.5	1085
Median	1167	1724
75% Percentile	1648	2004
Maximum	1781	2251

Table Analyzed Macrophages-CD86	Active	Inactive
Number of values	6	8
Mean	1120	1579
Std. Deviation	543.1	606.2
Std. Error	221.7	214.3

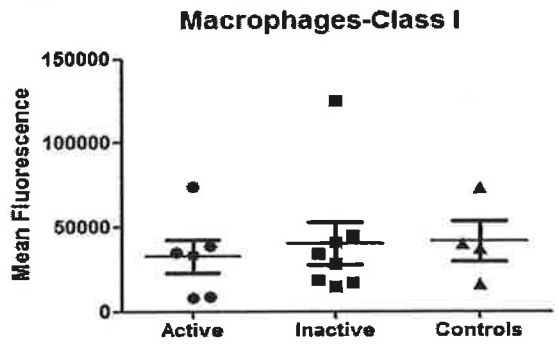
Macrophages-CD86	Active vs Inactive
Mann Whitney test	
P value	0.1419
P value summary	ns

Fig 3.1.14: A: Macrophages CD86 levels of mean fluorescence from patients with active HSK and after treatment. B: Comparison of Mean fluorescence data for all active and inactive patients and healthy controls. C: Statistical information for Graph B including means and median and a Mann-Whitney test to compare data.

A



B



C

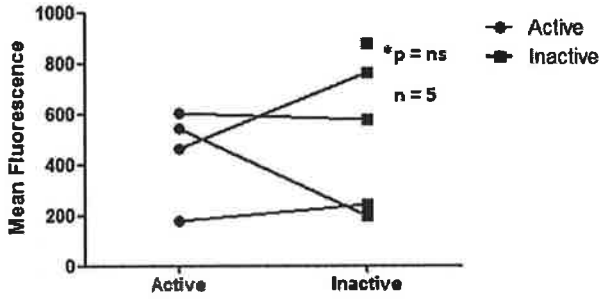
Table Analyzed Macrophages-Class I	Active	Inactive
Number of values	6	7
Minimum	7933	15120
25% Percentile	8733	16813
Median	34008	28524
75% Percentile	47457	40694
Maximum	73883	44487

Table Analyzed Macrophages-Class I	Active	Inactive
Number of values	6	7
Mean	32913	28309
Std. Deviation	24127	11899
Std. Error	9850	4497

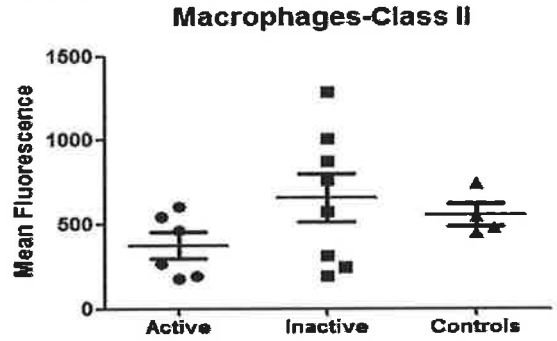
Macrophages-Class I	Active vs Inactive
Mann Whitney test	
P value	1.0000
P value summary	ns

Fig 3.1.15: A: Macrophages MHC Class I levels of mean fluorescence from patients with active HSK and after treatment. B: Comparison of Mean fluorescence data for all active and inactive patients and healthy controls. C: Statistical information for Graph B including means and median and a Mann-Whitney test to compare data.

A



B



C

Table Analyzed Macrophages-Class II	Active	Inactive
Number of values	6	8
Minimum	180	196
25% Percentile	190.5	260.3
Median	366.5	670
75% Percentile	559.3	974.5
Maximum	605	1290

Table Analyzed Macrophages-Class II	Active	Inactive
Number of values	6	8
Mean	376	658.1
Std. Deviation	184.9	394.7
Std. Error	75.5	139.6

Macrophages-Class II	Active vs Inactive
Mann Whitney test	
P value	0.1419
P value summary	ns

Fig 3.1.16: A: Macrophages MHC Class II levels of mean fluorescence from patients with active HSK and after treatment. B: Comparison of Mean fluorescence data for all active and inactive patients and healthy controls. C: Statistical information for Graph B including means and median and a Mann-Whitney test to compare data.

3.2 Comparative analysis of levels of TLR-induced TNFα, IL-6 and Rantes expression from Human Peripheral Blood Mononuclear cells derived from active and inactive HSK patients

In this study we wanted to examine the levels of our selected cytokines and chemokines from cells isolated from patients suffering from HSV-induced keratitis compared with the same patients having presented the virus cleared from the system and having been clinically diagnosed as having inactive disease. This analysis was done by ELISA as described in the material and methods part of this thesis. The levels of cytokine production should give us a clearer picture of how the immune system of the HSK patients is responding to HSV infection. Statistical analysis of the results was performed using a non parametric paired t test and below (figure 3.2.1) is the legend used to annotate the various graphs.

Pvalue	Wording	Summary
< 0.001	Extremely significant	***
0.001 to 0.01	Very significant	**
0.01 to 0.05	Significant	*
>0.05	Not significant	ns

Fig 3.2.1: Legend of comparison of ELISA dot plots Figs 3.2.2 – 3.2.9

***Note: 0 time point represents unstimulated cells.**

Tumour necrosis factor alpha (TNFα) levels were analysed in cells derived from the different patient population. A fold increase in TNFα production was detected following Lipopolysaccharide (LPS) treatment of peripheral blood mononuclear cells (PBMCs) for 24 hours which was statistically significant change from the resting cells. When inactive and active patient PBMC responses were compared.(fig 3.2.2a) these altered responses indicate that the infected cells may be less responsive to LPS, these differences were not found to be statistically significant between the active and inactive cells ($p>0.05$). Interestingly the LPS responses of the control PBMCs indicated that they are behaving more like the active patient samples, with reduced ability of TLR4 stimulation to drive TNFα production. In general higher levels of TNFα was observed in imiquimod stimulated cells, although responses to TLR7 stimulation were modest they were higher in the patient cell population compared to healthy controls (fig3.2.2b)

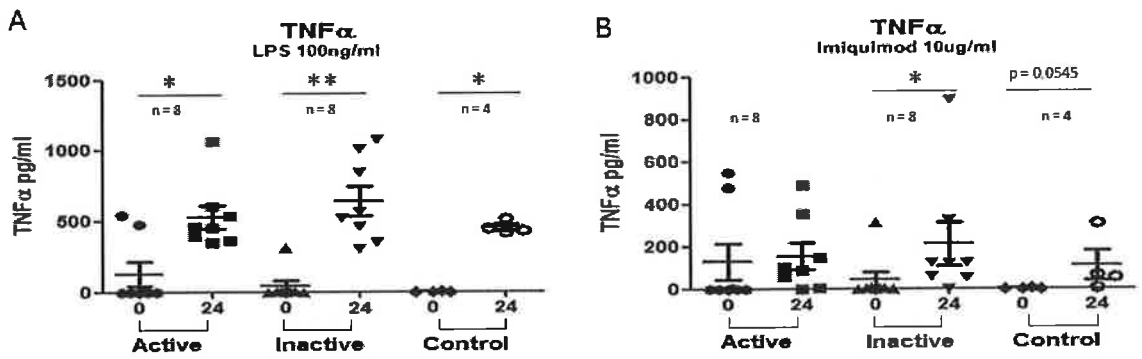


Fig 3.2.2: A: TNFα levels were measured by ELISA in human peripheral mononuclear cells of patients with active HSK, inactive HSK and a healthy control. Cells were plated at 2.5 x10^5 cells/ml and stimulated with A: Lipopolysaccharide (LPS). B: Imiquimod.

Analysis of levels of TNFα detected in PBMC samples treated with the TLR9 agonist CpGa derived from active HSK patients compared to either inactive samples or those derived from healthy controls showed no increase in levels of TNFα (figure 3.2.3a). There were slight increases in TNFα production for TLR3-treated PBMCs after 24 hrs but no significant differences between active and inactive (Figure 3.2.3b). The healthy controls did not respond at all to TLR3 stimulation perhaps indicating that HSK patients are more sensitive to TLR3 stimulation. (Figure 3.2.3b).

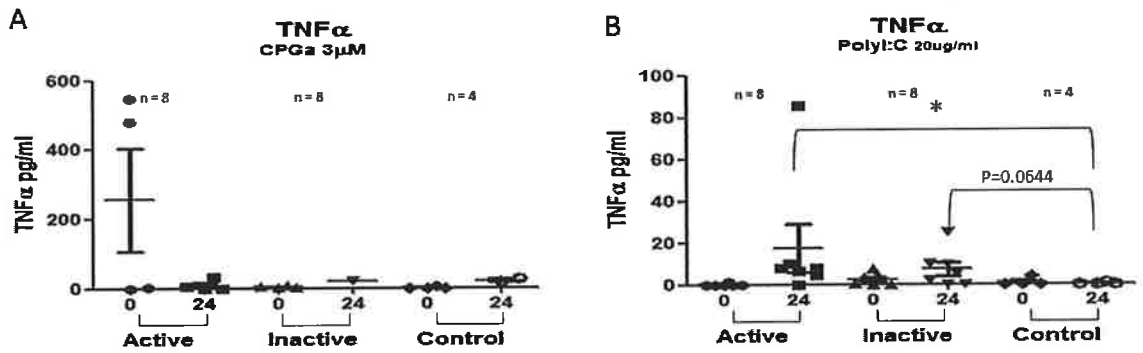


Fig 3.2.3: A: TNFα levels were measured by ELISA in human peripheral mononuclear cells of patients with active HSK, inactive HSK and a healthy control. Cells were plated at 2.5 x10^5 cells/ml and stimulated with C: CpG Oligodeoxynucleotide Class A (CPGa) and D: polyinosinic-polycytidylic acid (poly I:C).

Regarding IL-6 production, again little difference was seen between resting cell populations (figure 3.2.4a and 3.2.4b). Both active and inactive HSK samples responded to LPS to the same extent as healthy control samples. All of these responses were statistically significant ($p< 0.05$), Interestingly however, CpGa stimulation of the cells profoundly altered the ability of PBMCs from active HSK patients to respond to this TLR9 stimulus (figure 3.2.5a), whereas some IL-6 was induced following CpGa treatment of PBMCs derived from active patients compared with inactive and healthy controls indicating that TLR9 may be over responding in active patients but with high variance between samples more data is needed to confirm this observation. Responses to TLR7 were the same across all sample groups with TLR7 stimulation inducing IL-6 expression in all cases to the same extent (figure 3.2.4b). Interestingly responses to TLR3 stimulation were slightly raised in the active PBMCs compared with inactive or control samples (figure 3.2.5b), suggesting that immune cells are primed to drive IL-6 stimulation in response to certain TLR ligands.

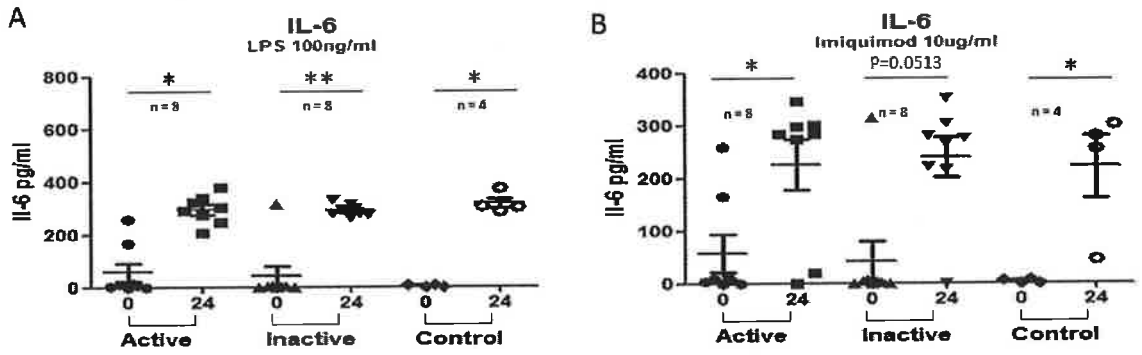


Fig 3.2.4: A: IL-6 levels were measured by ELISA in human peripheral mononuclear cells of patients with active HSK, inactive HSK and a healthy control. Cells were plated at 2.5 x10^5 cells/ml and stimulated with A: Lipopolysaccharide (LPS). B: Imiquimod,

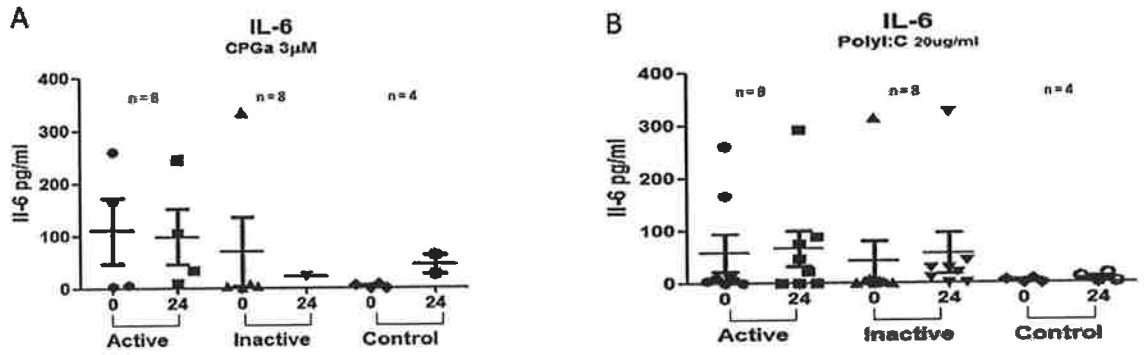


Fig 3.2.5: A: IL-6 levels were measured by ELISA in human peripheral mononuclear cells of patients with active HSK, inactive HSK and a healthy control. Cells were plated at 2.5 x10^5 cells/ml and stimulated with C: CpG Oligodeoxynucleotide Class A (CPGa) and D: polyinosinic-polycytidylic acid (poly I:C).

When the levels of the chemokine Rantes were examined, it appeared levels were elevated in resting cells derived from active patients compared with inactive patients. Interestingly, whilst TLR4 and TLR7 stimulation resulted in a slightly elevated production of Rantes between active and control samples compared with inactive samples (figure 3.2.6a & b). The differences were statistically significant ($p< 0.05$) only between inactive patients and healthy control at no stimulation and 24 hours). CpGa stimulation of TLR 9 resulted in an increase in Rantes production in active samples which is comparable with inactive and healthy controls. (Figure 3.2.7a). TLR 3 stimulated Rantes was slightly raised in the inactive patients but mostly there was no observable response (figure 3.2.7b).

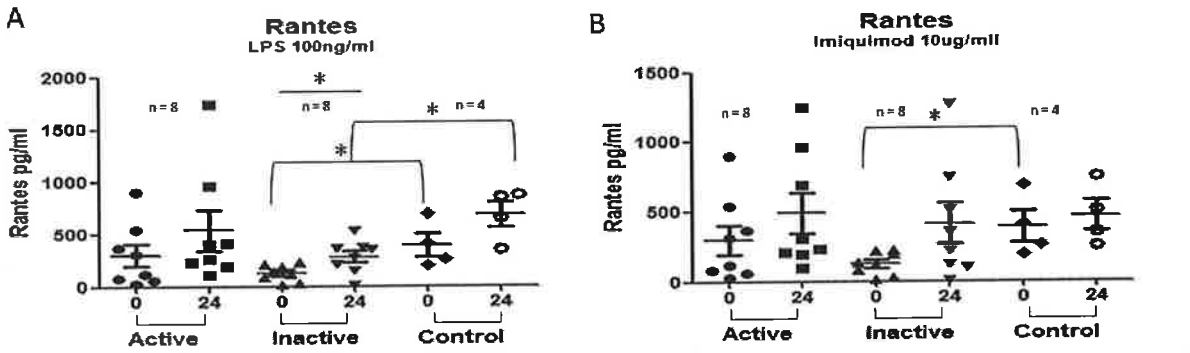


Fig 3.2.6: A: Rantes levels were measured by ELISA in human peripheral mononuclear cells of patients with active HSK, inactive HSK and a healthy control. Cells were plated at 2.5 x10^5 cells/ml and stimulated with A: Lipopolysaccharide (LPS). B: Imiquimod,

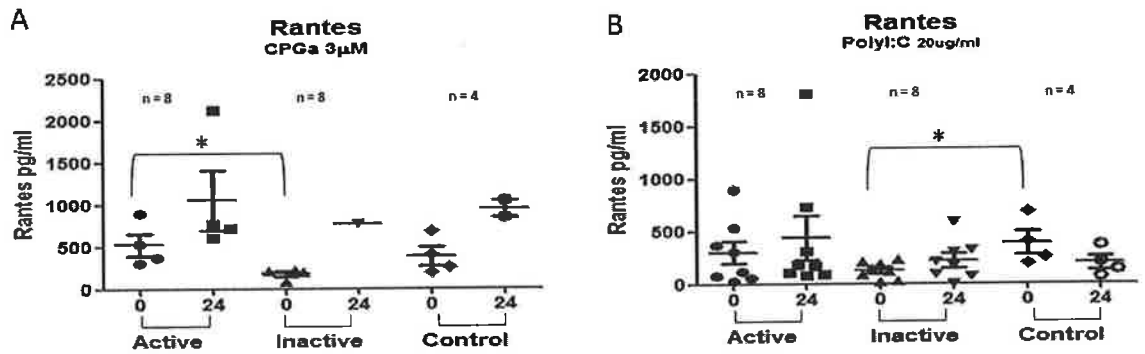


Fig 3.2.7: A: Rantes levels were measured by ELISA in human peripheral mononuclear cells of patients with active HSK, inactive HSK and a healthy control. Cells were plated at 2.5 x10^5 cells/ml and stimulated with C: CpG Oligodeoxynucleotide Class A (CPGa) and D: polyinosinic-polycytidylic acid (poly I:C).

With respect to IFNγ production, unfortunately, in all cases the amount of IFNγ produced in response to all stimuli was below the level of detection (figure 3.2.8 and 3.2.9). Thus we were unable to make any conclusions from this data.

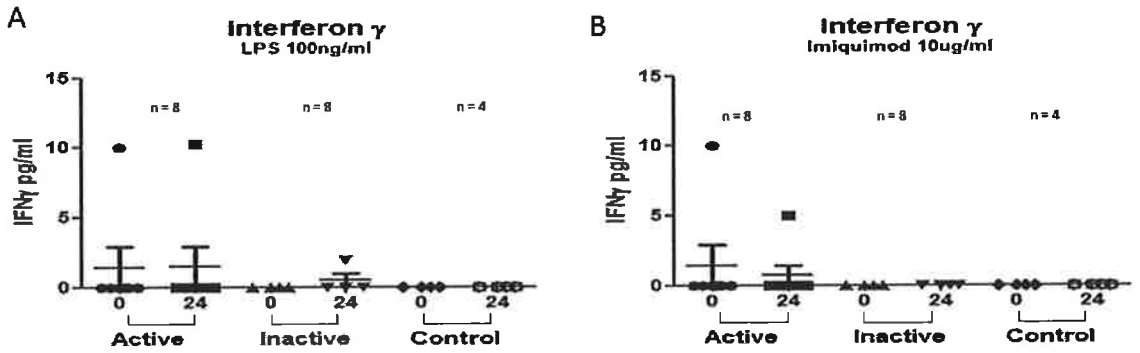


Fig 3.2.8: A: IFNγ levels were measured by ELISA in human peripheral mononuclear cells of patients with active HSK, inactive HSK and a healthy control. Cells were plated at 2.5 x10^5 cells/ml and stimulated with A: Lipopolysaccharide (LPS). B: Imiquimod.

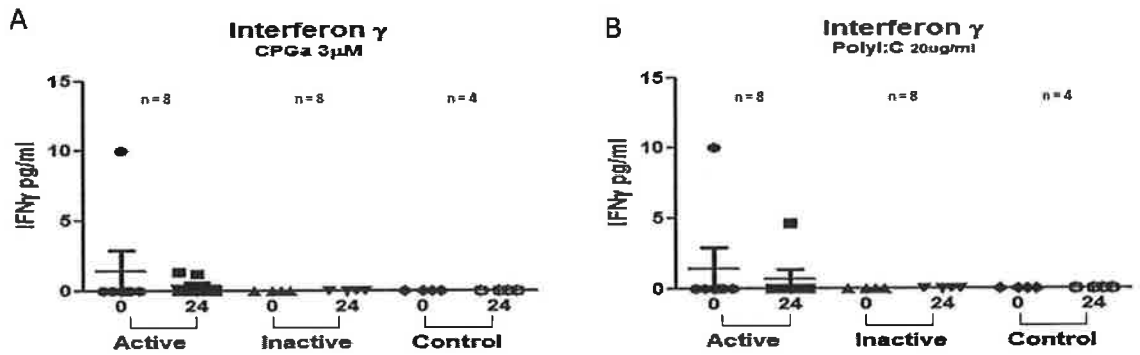


Fig 3.2.9: A: IFNγ levels were measured by ELISA in human peripheral mononuclear cells of patients with active HSK, inactive HSK and a healthy control. Cells were plated at 2.5 x10^5 cells/ml and stimulated with C: CpG Oligodeoxynucleotide Class A (CPGa) and D: polyinosinic-polycytidylic acid (poly I:C).

3.3 Analysis of proinflammatory cytokines in human plasma with HSK, before and after treatment.

Meso scale discovery (MSD) multi array is a multiplex immunoassay system that enables the measurement of biomarkers using the next generation of electroluminescent detection technology. It generates minimal background, analyses seven cytokines in one go and uses minimal sample. The MSD plate used in this study analyses the following cytokines and chemokines: Interleukin 1β, IL-12p70, Interferon γ, Interleukin-6 (IL-6), Interleukin-8 (IL-8), Interleukin-10 (IL-10), Tumour Necrosis Factor α. MSD plates have high sensitivity up to five logs linear dynamic range and is much more sensitive than traditional ELISA techniques.

When IL-1β was analysed elevated levels of IL-1β were detected in active patient sera compared with inactive, with a further reduction observed in serum derived from healthy controls. The active Vs the inactive and the active Vs control were statistically significant ($p < 0.05$), $p < 0.001$). This observation shows that there is inflammation in the body caused by a viral infection in the eye which is detectable in PBMC's. A line graph linking each patient sample shows a trend towards decreasing levels of IL-1β which shows that levels are returning to normal after HSK patients have undergone treatment and are inactive but these levels are still slightly higher than the control samples.

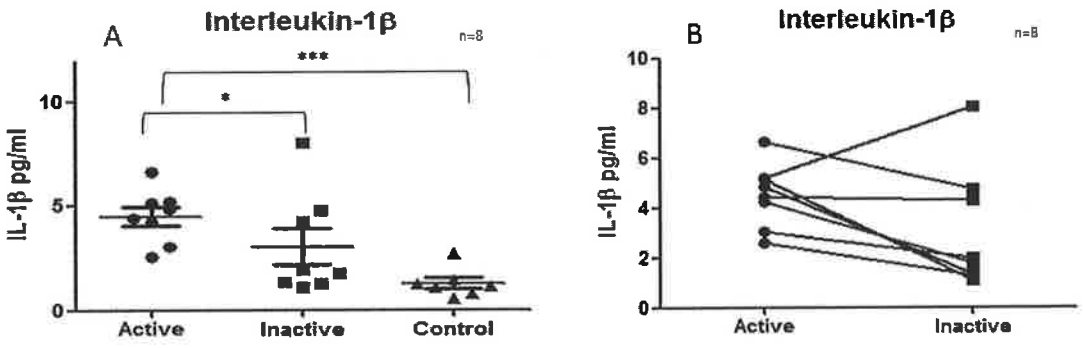


Fig 3.3.1: Interleukin 1β levels were measured by MSD in human plasma from patients with active HSK and after treatment inactive and healthy controls. A: Scatter plot of Active against inactive disease. B: Line graph showing levels of individual patients before and after treatment.

Interleukin-12p70 consists of p40 and p35 subunits. It stimulates the secretion of interferon γ and TNF α and inhibits IL-4 proliferation of lymphocytes. Interestingly we observed that active patients had less circulating IL-12/p70 when compared with either patients with inactive disease or healthy controls (figure 3.3.2a). This suggests that IL-12 which is important for Type II interferon production is perhaps being inhibited by the virus. A line diagram linking patients before and after treatment confirms a trend towards increasing levels of IL-12 (Figure 3.3.2b).

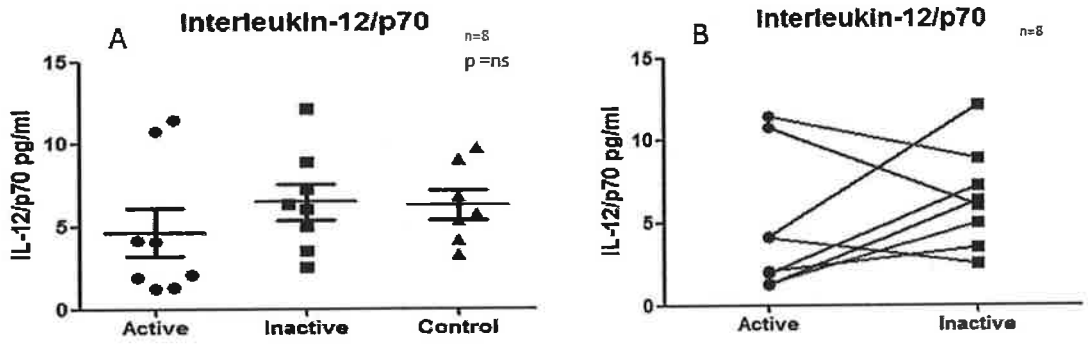


Fig 3.3.2: Interleukin 12/p70 levels were measured by MSD in human plasma from patients with active HSK and after treatment. A: Scatter plot of active against inactive disease. B: Line graph showing levels of individual patients before and after treatment.

For IL-8, no trend towards increased serum levels of IL-8 in active patients was observed (Figure 3.3.3). Reduced IL-10 levels were observed in active patient samples (figure 3.3.4), suggesting less immunosuppressive or immune modulatory effects as a result. For all other cytokines measured, IFNγ, IL-6, TNFα etc. no observable differences in serum cytokine levels between active and inactive patients were observed (figures 3.3.5-3.3.7).

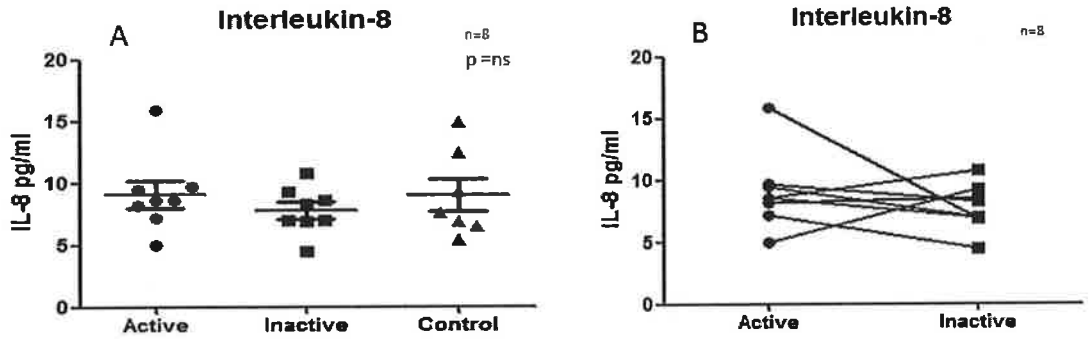


Fig 3.3.3: Interleukin-6 levels were measured by MSD in human plasma from patients with active HSK and after treatment. A: Scatter plot of Active against inactive disease. B: Line graph showing levels of individual patients before and after treatment.

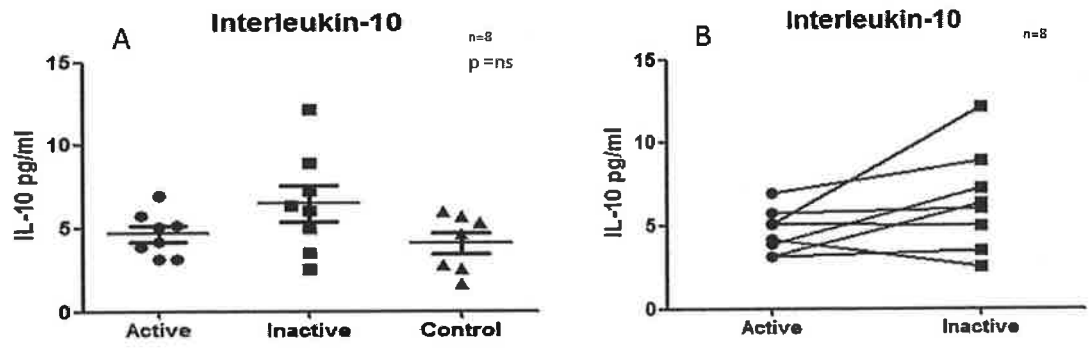


Fig 3.3.4: Interleukin-10 levels were measured by MSD in human plasma from patients with active HSK and after treatment. A: Scatter plot of Active against inactive disease. B: Line graph showing levels of individual patients before and after treatment.

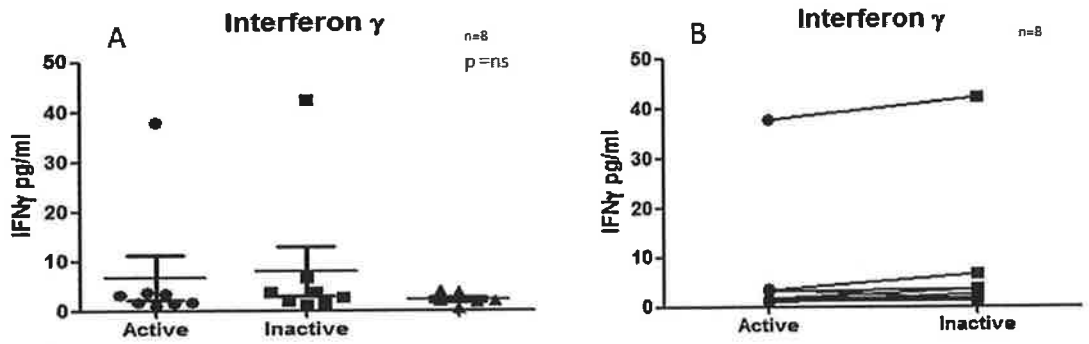


Fig 3.3.5: Interferon γ levels were measured by MSD in human plasma from patients with active HSK and after treatment against healthy controls. A: Scatter plot of active against inactive disease. B: Line graph showing levels of individual patients before and after treatment.

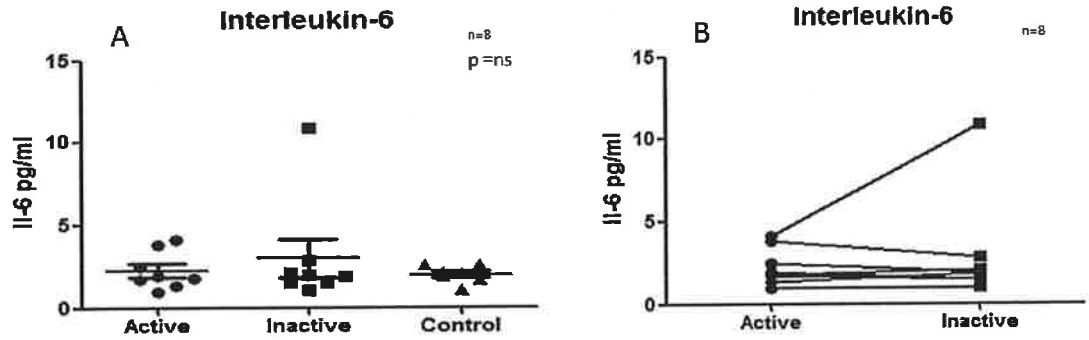


Fig 3.3.6: Interleukin-6 levels were measured by MSD in human plasma from patients with active HSK and after treatment. A: Scatter plot of active against inactive disease. B: Line graph showing levels of individual patients before and after treatment.

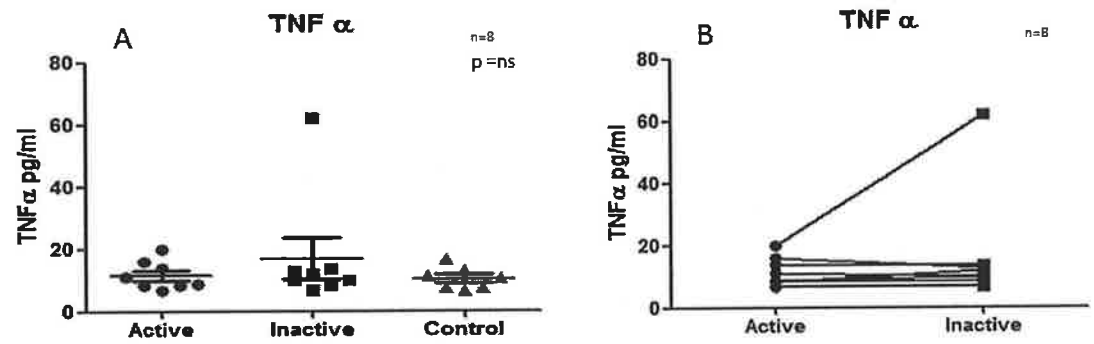


Fig 3.3.7: TNFα levels were measured by MSD in human plasma from patients with active HSK and after treatment. A: Scatter plot of active against inactive disease. B: Line graph showing levels of individual patients before and after treatment.

3.4 Differences in IRF3 and STAT 1 levels in peripheral blood mononuclear cells of HSK active patients and after treatment.

As IFN production and IFN signalling are known to be perturbed in cells in HSV infected cells, we set out to ascertain how HSV-1 affects the innate immune system in PBMCs by measuring changes in levels of IRF-3 and STAT-1 by western blotting.

Western blot analysis of resting PBMCs from active and inactive patients demonstrated that there is a trend towards higher levels of IRF3 and Stat1 in the inactive patient samples compared with the active (figure 3.3.1). This might be expected as in active disease IRF3 and Stat1 are being used to signal transcription of interferons for release and to help in viral clearance and are thus targeted by HSV for degradation. These differences were not statistically significant when compared using a non parametric t test. ($p > 0.05$)

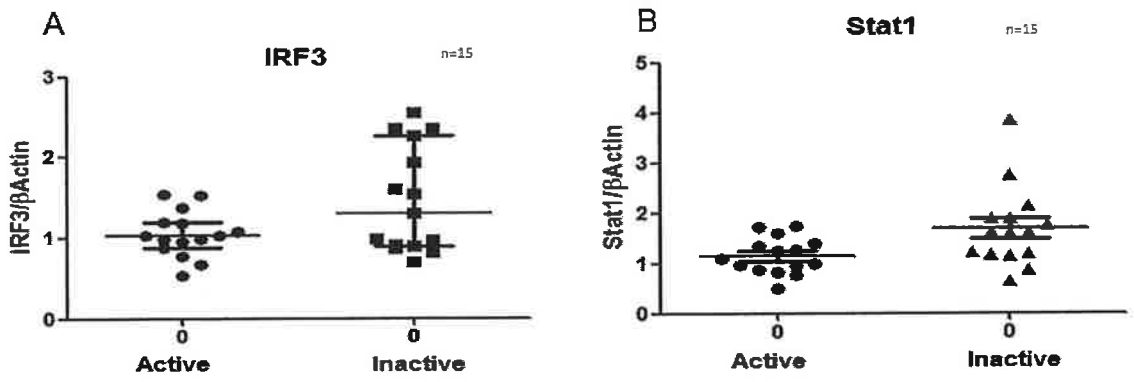
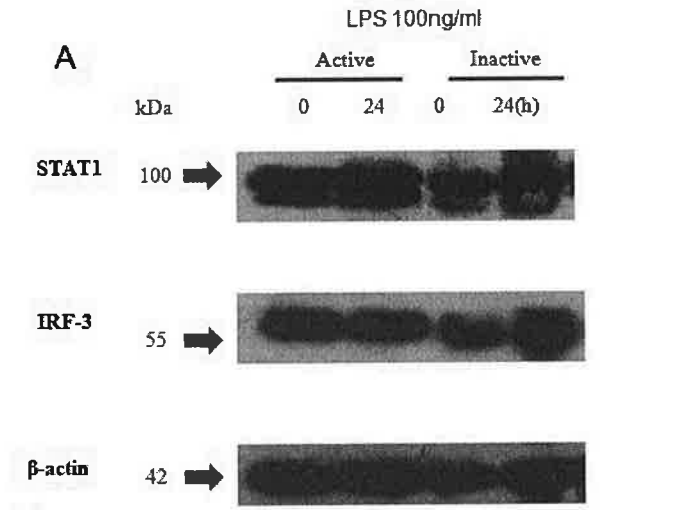
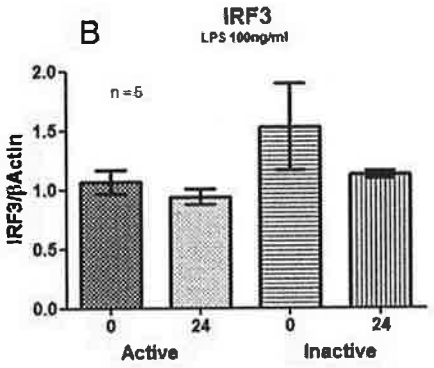
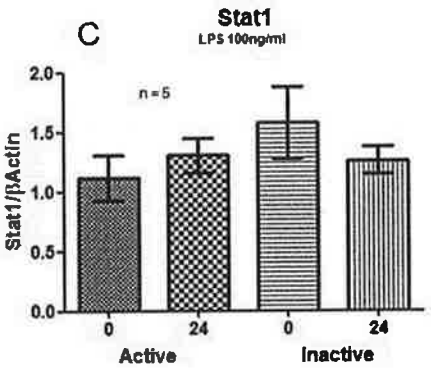


Fig 3.4.1 : IRF3 and Stat 1 levels were measured by western blot in human peripheral mononuclear cells plated at 1 x10^6 cells/ml. Cells were lysed and levels of total A: IRF3 and B: Stat1 were analysed by western blot

To ascertain how HSV-1 affects the innate immune system in PBMCs, changes in levels of protein for STAT1 and IRF-3 were determined following TLR stimulation of PBMCs by western blotting. Both for IRF3 (figure 3.4.2b) and STAT1 (Figure 3.4.2c), enhanced resting levels of both proteins was observed in inactive PBMC samples. Following stimulation of PBMCs with LPS (Figure 3.4.2), a reduction in for IRF3 (figure 3.4.2b) and STAT1 (Figure 3.4.2c) was observed in inactive PBMC samples, whilst no change was observed in PBMCs derived from active patients.







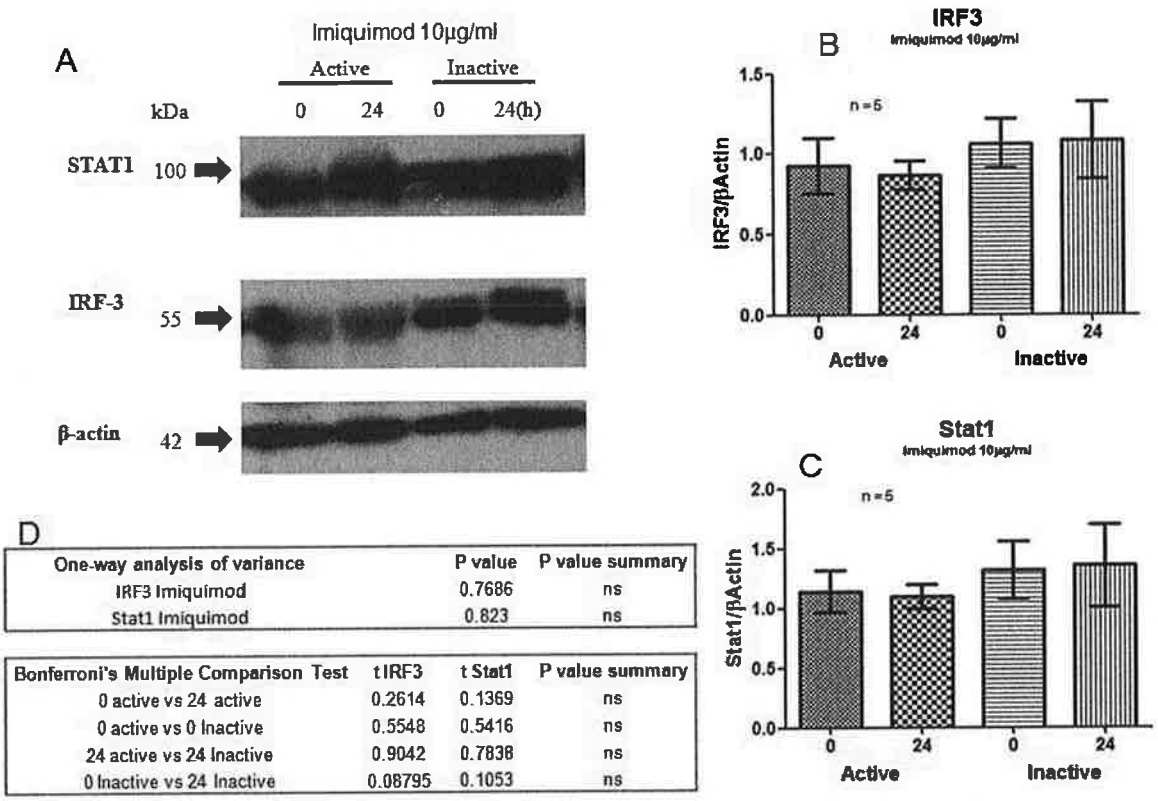
D

One-way analysis of variance	P value	P value summary
IRF3 LPS	0.1911	ns
Stat1 LPS	0.4566	ns

Bonferroni's Multiple Comparison Test	t IRF3	t Stat1	P value summary
0 active vs 24 active	0.4779	0.6469	ns
0 active vs 0 Inactive	1.71	1.613	ns
24 active vs 24 Inactive	0.7001	0.1564	ns
0 Inactive vs 24 Inactive	1.488	1.123	ns

Fig 3.4.2: IRF3 and Stat 1 levels were measured by Western Blot in human peripheral mononuclear cells plated at 1 x10^6 cells/ml and stimulated with Lipopolysaccharide. A: Representative blots of Stat 1 IRF 3 and loading control β-Actin. B: Combined IRF3 densitometry data of n=5 patients. C: Combined Stat 1 densitometry data of n=5 patients. D: Statistical data of densitometry analysis of blots.

When PBMC responses to imiquimod (TLR7 stimulation) were analysed, no observable changes in either IRF3 (figure 3.4.3b) or STAT1 (figure 3.4.3a) were observed.



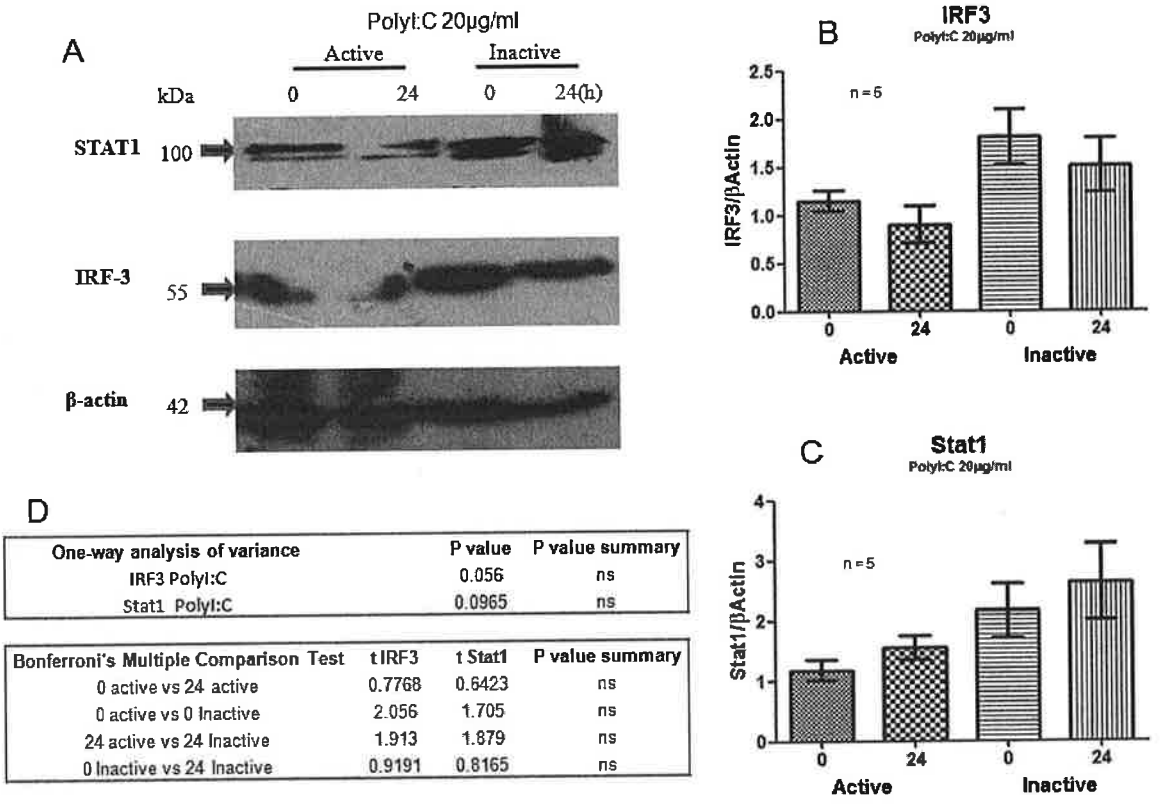
D

One-way analysis of variance	P value	P value summary
IRF3 Imiquimod	0.7686	ns
Stat1 Imiquimod	0.823	ns

Bonferroni's Multiple Comparison Test	t IRF3	t Stat1	P value summary
0 active vs 24 active	0.2614	0.1369	ns
0 active vs 0 Inactive	0.5548	0.5416	ns
24 active vs 24 Inactive	0.9042	0.7838	ns
0 Inactive vs 24 Inactive	0.08795	0.1053	ns

Fig 3.4.3: IRF3 and Stat 1 levels were measured by Western Blot in human peripheral mononuclear cells plated at 1 x10^6 cells/ml and stimulated with Imiquimod. A: Representative blots of Stat 1 IRF 3 and loading control β-Actin. B: Combined IRF3 densitometry data of n=5 patients. C: Combined Stat 1 densitometry data of n=5 patients. D: Statistical data of densitometry analysis of blots.

However TLR3 stimulation of PBMCs derived from active and inactive patients demonstrated that polyI:C drove the degradation of IRF3 in both cases (Figure 3.4.4b) whereas STAT1 levels were enhanced in the same samples (Figure 3.4.4c). The differences in absolute levels of IRF3 and STAT1 between active and inactive patients following TLR3 stimulation would suggest that PBMCs derived from active patients would be able to produce much less type I interferon than inactive PBMCs thus enhancing viral survival in the host.



D

One-way analysis of variance	P value	P value summary
IRF3 Polyl:C	0.056	ns
Stat1 Polyl:C	0.0965	ns

Bonferroni's Multiple Comparison Test	t IRF3	t Stat1	P value summary
0 active vs 24 active	0.7768	0.6423	ns
0 active vs 0 Inactive	2.056	1.705	ns
24 active vs 24 Inactive	1.913	1.879	ns
0 Inactive vs 24 Inactive	0.9191	0.8165	ns

Fig 3.4.4 : IRF3 and Stat 1 levels were measured by Western Blot in human peripheral mononuclear cells plated at 1 x10^6 cells/ml and stimulated with polyinosinic-polycytidylic acid (poly I:C). A: Representative blots of Stat 1 IRF 3 and loading control β-Actin. B: Combined IRF3 densitometry data of n=5 patients. C: Combined Stat 1 densitometry data of n=5 patients. D: Statistical data of densitometry analysis of blots.

4. Discussion

Herpes Simplex Keratitis is a common cause of vision loss, particularly in recurrent disease where corneal stromal scarring, irregularity and vascularisation are likely to occur. In many cases the ensuing visual morbidity requires corneal transplantation which, in the context of HSK, has a poor long term success rate. There is therefore a significant need to develop new treatments which reduce inflammation-induced damage to the cornea and the recurrence rate in the long term. Treatments that inhibit HSV replication in the cornea and reduce the intensity of the inflammatory response that is responsible for corneal opacification would of great benefit to patients. Clearly, we need to improve our understanding of how the immune response of HSK patients differs during the active phase of disease compared to when it is quiescent and compared to healthy controls as a step towards this goal.

The aim of this study was to examine immune status and responsiveness of peripheral blood mononuclear cells of patients with HSK and to determine if we could identify markers of disease activity and examine the changes that occur from when a patient presents with active HSK disease to their return visit where all symptoms and signs have cleared and they are diagnosed as inactive. It is useful to look at these changes to see which cells are responding in the immune response to HSV and which cells might have a compromised response perhaps caused by a mechanism used by HSV-1 to bypass the normal immune functions to begin replication. To mount an efficient response to infection the cell must activate a number of pathways, including Nuclear Factor kappa β (NF-κβ), Interferon Regulatory Factors (IRFs) and Mitogen-Activated Protein Kinase pathways. HSV-1 blocks the activity of Interferon Stimulated genes (ISGs) including the protein Kinase R and the 2'-5'-OAS/RNAse L (2'-5- A) system. IFN α/β in conjunction with IFN γ provide the primary resistance to HSV infection and the virus diminishes Interferon production through several viral proteins including ICP0, ICP27, ICP34.5 and vhs (Melchjorsen, Matikainen et al. 2009).

To look at the disease state we examined the activation markers on T cells, B cells, NK cells and Macrophages by flow cytometry. We looked at patients in the active state and the inactive state and we also looked at similar markers on the immune cells of healthy controls. In the immune cells of a virally infected patient we would expect to see differences in these

activation markers. Also we would expect to see recruitment of MHC Class I and II for activity with T cells and NK cells .Although not statistically significant, our findings showed a trend towards enhanced expression activation of the CD69 activation marker on $CD8^+$ T cells, $CD4^+$ T cells and in NK cells. This gives a clear indication that the immune system of the active patients are activated when compared to the level of activation measured after they were diagnosed as being inactive and compared to healthy control donors. This also validates that we are looking at the immune cells of patients with an active disease form. Some of the inactive samples gave high levels of CD69 but this may be attributed to the fact that although their HSK symptoms such as inflammation, redness had disappeared and any ulcers had healed, their immune system still had not returned to an inactive state. In contrast CD25, the alpha chain of the IL-2 receptor found on activated T cells showed no change between active, inactive and healthy controls. however the lack of statistical significance and the spread of readings across active samples suggests that peripheral T cell and NK cell activation may not be apparent in HSK and that active $CD8^+$ T cells and NK cells will be found at the site of infection as has been previously demonstrated by other groups (Tamesis, Messmer et al. 1994; Lepisto, Frank et al. 2006).

In B cells and macrophages we noticed that CD80 an activation marker for B cells and monocytes demonstrated some degree of activation in B cells, but not in monocytes. CD86 another marker on B cells and monocytes for activation that works in tandem with CD80 to prime T cells showed no changes in expression in either B cells or monocytes across patient samples. Interestingly expression of Major histocampatibility complexes (MHC) I and II were unaltered on B cells. However on monocytes a trend indicating a decrease in MHC expression was observed, particularly for class II. MHC class II role in the immune response to HSV-1 might be compromised, as might the clearance of HSV-1 from the corneal epithelium. It has been shown that HSV infected cells stimulate the production of $CD4^+$ T cells through the MHC class II pathway but HSV diminishes the activity of MHC class II by the viral envelope glycoprotein B (Neumann, Eis-Hubinger et al. 2003). The number of patients in this study was not large enough to make conclusions on this and differences were statistically non-significant ($p>0.05$). The recruitment of a larger number of patients as well as a study examining cell responses and activations to ligands on cells that showed specific

trends would need to be conducted to validate these conclusions. A sample as large as 100 would allow immune response variances and different disease states to be accounted for.

Cytokine production is of particular importance in virally infected cells. Once a virus is detected in the body a stress response is triggered and different cells such as lymphocytes release anti viral cytokines to try and combat the virus by triggering cell death. In studies TNFα was shown to have a protective effect against HSV-1 infection in mice independent of interferons (Rossol-Voth, Rossol et al. 1991). Tumour Necrosis Factor α (TNFα) plays a key role in the acute phase reaction and systemic inflammation. It is mainly produced by macrophages but can be produced by other cells under pathogenic conditions. TNFα drives a number of cell signalling pathways including apoptosis, proliferation, chemo-attraction and cytokine production. TNFα can also contribute to tumorigenesis and viral replication.

In this study while TNFα production after TLR4 stimulation for 24 hours appeared to be normal in both active and inactive patient samples, and we saw a marked response for healthy controls. One interesting difference was the response in the inactive cells after TLR 4 stimulation, which appeared to give a scattered response and a larger spread than the active patients. This erratic response might simulate what happens in active patients as perhaps their immune system hyper-responds to early viral infection releasing more TNFα causing infiltration of inflammatory cells to the site of infection which contribute to the symptoms of HSK but do not do an effective job in clearing the virus. In the previously mentioned study there was a marked rise in activation of NK cells in the mice. An analysis of a larger patient population would perhaps give more of an insight into whether or not this is happening. With TLR 7 stimulation there was a slight increase of TNFα in HSK patients cells response compared to healthy controls. This observation was not statistically significant and an increase of patient numbers would be required to see if this trend is true. TLR 9 stimulation did not see any marked increase in TNFα levels but TLR 3 stimulation shows a significant difference between active and healthy controls and a difference approaching significance between inactive cells and healthy controls. This again suggests that HSK sufferers produce more TNFα upon viral infection.

Interleukin 6 is proinflammatory cytokine secreted by monocytes and macrophages in response to tissue damage or infection. It plays a role in the acute phase response the regulation of fever and the generation of plasma B cells. HSK patient cells upon stimulation of TLR 4, 7 and 9 show normal responses to IL-6 production compared with healthy controls as a significant increase was observed from the resting state. TLR 3 stimulation shows an observable increase in IL-6 production in patient cells both active and inactive compared to healthy controls, but these differences were not statistically significant. In a study with mice that were infected with UV-inactivated HSV-1 that was incapable of expressing and maintaining high levels of IL-6 HSK failed to develop (Kanangat, Babu et al. 1996). This suggests that IL-6 plays a part in the inflammation in the cornea of HSK but this is after the HSV-1 virus has evaded other immune responses which prevent the HSV-1 virus from reactivating in the corneas of patients who suffer from recurrent HSK.

Rantes or CCL5 helps recruit leucocytes to the site of infection. In studies it has been shown that HSV induces Rantes production through transcription factors NF-κβ and IRF-3. Inhibition of these pathways can drastically reduce Rantes production. (Melchjorsen and Paludan 2003). In patient samples with TLR 3, 4 and 7 stimulated individually there appears to be an observable decrease in Rantes levels in the active and inactive patient cells. This could signify some interference with either NF-κβ or IRF-3 pathways therefore reducing Rantes production and recruitment of T cells and other leucocytes to the site of infection. This observation would fit with the reduction we observed in both IRF-3 and STAT1 levels in PBMCs of active patients by western blotting, suggesting that IRF-3 signalling and function is perturbed. The reduction of leukocyte recruitment may be a good thing as this transport of cells to the immune privileged cornea is responsible for some of the complications of HSK. There is a statistically significant difference between inactive and healthy controls in TLR4 stimulated cells and unstimulated cells. A further study recruiting more patients could confirm these findings as well as a more in-depth study on the various pathways.

Cytokine production was also analysed in the plasma serum of patients. This allows us to see what levels are being produced by the immune cells in active and inactive patient serum and compare it to healthy controls. The analysis was done by Meso Scale Discovery (MSD) as it is

more sensitive than traditional ELISA and we are measuring differences in basal levels which may be quite low.

Firstly we looked at Interleukin 1β which is produced by dendritic cells, monocytes and macrophages. During the course of infection it has both local and systemic effects and elevated levels of serum IL-1β is indicative of either infection or inflammatory disease. After HSV infection of the cornea the most prominent cytokine that are produced are IL-6 and IL-1 which result after several days in stromal lesions, this has been shown in murine models in vivo and in excised mouse corneas (Staats and Lausch 1993; Lausch, Chen et al. 1996). From our results we saw a statistically significant change from the active patient cells to the inactive form. This active change was also more significant when compared to healthy controls. So HSV causes upregulation of IL-1β, potentially contributing to the recruitment of leucocytes to the site of infection thus causing some of the damage to the corneal stroma and can lead to vascularisation as the body struggles to deliver these cells to the site of infection. A clear trend towards decreased IL-1β can be observed after the patients have undergone treatment. IL-1β is enhanced in many diseases caused by viruses and the targeting of these pathways to reduce over production of this cytokine could potentially reduce the severity of the HSK.

Next we looked at Interleukin-12p70 which consists of p40 and p35 subunits. It stimulates the secretion of interferon γ and TNF α and inhibits IL-4 proliferation of lymphocytes. Studies have shown that HSV-1 infection in a mouse model yielded sustained expression of IL-12. This local production caused an immunopathological response in the mouse eye due to Th1 immunity. (Hendricks, Janowicz et al. 1992). In the cytokine profile of HSK active patients there is an observable trend towards decreased levels of IL-12 when compared with the Inactive patients and the healthy controls. This lack of IL-12 during early infection may contribute to HSV-1 being able to establish itself in the cornea for longer periods as IL-12 plays a role in interferon production especially type II interferons. In this study we did look at Interferon γ, a type II interferon which plays a role in the recruitment of leukocytes to the site of infection but more importantly increases the effectiveness of type I interferons towards viral clearance. IFN-γ is produced by Th1 cells and NK cells. IFN-γ activates

macrophages by increasing the expression of major histocompatiblity complex MHC molecules. It also enhances the effect of type 1 interferons. Unfortunately none of our analyses were able to detect IFN γ meaning either it is not being produced or it is lower than the detection limits of our tests. We stimulated TLR 3, 4, 7 and 9 in cells for 24 hours and also tried to detect levels using the MSD assay which is a more sensitive technique. IFN γ appears to play a vital role in viral clearance and its presence or lack thereof would be of interest to this project.

For the final part of this study we wanted to examine some of the pathways that follow TLR stimulation, through a series of activation factors and phosphorylation leading to transcription factors entering the nucleus, followed by the production of more cytokines, chemokines or antiviral agents such as interferons. For this study we examined IRF3 and Stat1. Interferon Regulatory Factors (IRFs) are a family of related transcription proteins that act as regulators of the Interferon α/β gene promoters. Relative levels of IRF3 mRNA do not change in virus infected cells but a study showed that IRF3 does degrade over time in virally infected cells as interferons are produced to combat the infection (Lin, Heylbroeck et al. 1998). When we looked at total IRF3 in active patient cells compared to inactive patients we do see a lower amount in the active cells. This suggests that IRF3 in the active patient cells is being used to activate the transcription of interferons α/β. When we stimulated TLRs 3,4 and 7 we observed a decrease in IRF3 levels over 24 hours for all three stimulations in both active and inactive patient cells suggesting that IRF3 is behaving as expected in HSK patient cells suggesting that they are producing interferons. In studies it has been shown that the HSV-1 protein ICP0 inhibits IRF3 mediated activation of interferons but these observations were in HEK 293 cells and not Human PBMCs (Lin, Noyce et al. 2004).

To further examine the production of Interferons in the cells of HSK patients signal transducer and activator of transcription (Stat1) levels were analysed in the cell lysates of patients before and after treatment. Once interferons are released from virus infected cells they bind to their cognate receptors on surrounding cells and through the Jak/Stat pathway Stat is phosphorylated and interacts with gene promoters in the nucleus to initiate transcription of more interferons including interferon γ. Stat 1 deficiency can result in

susceptibility to viral infection and studies have shown that fibroblasts in patients that are completely deficient in Stat1 production were not able to clear HSV replication when treated with IFN α/β but were able to clear some viruses in vivo (Chapgier, Wynn et al. 2006). In this study total Stat1 levels in unstimulated cells showed higher levels in the inactive patients sample. So similarly to IRF3 levels Stat1 seems to be degraded thus reducing its activity. Once active STAT1 is transported to the nucleus where it binds to the IFN stimulated response element (ISRE) within the promoter region of interferon stimulated genes (ISGs). For patient cells that had TLR 3, 4 and 7 stimulation we see no discernible pattern of changes in Stat1 emerging. For TLR 3 and 4 we see an increase in Stat1 over 24 hours in active cells. This might suggest that HSK active patients are not effectively producing type II interferons which work with type I interferons for viral clearance. The Stat 1 although it is being produced the virus is somehow inhibiting the phosphorylation events of the Jak/Stat pathway which is then reducing the production of interferon γ. However for TLR 7 we did not see an increase in Stat1 after 24 hours. Conversely in inactive patients there is a decrease in total Stat 1 for TLR 4 stimulated cells over 24 hours which might be expected. TLR 3 stimulation results in an increase in total Stat 1 implying that TLR 3 detection of pathogens results in a deficient response when type I interferons stimulate neighbouring cells for type II interferon production. This may be explained by the fact that TLR3 recruits only TRIF adaptor to signal IRF3 activation but TLR 4 can recruit MyD88 and TRIF and activates NF-κβ and AP-1 as well as IRF3 for Interferon production. Therefore if the MyD88 pathway is somehow compromised in HSK patients the TLR4 response will bypass this deficiency. TLR7 showed no change in total Stat1 production suggesting that TLR7 signalling is working but not as effectively as TLR4 signalling. TLR7 recruits MyD88 as well but activates IRF7 to induce the transcription of interferon genes. Similarly to IRF3 it has been shown that IRF7 transcription is inhibited by the HSV-1 virus. It has been shown in studies that Mice lacking the Stat1 signalling molecule are more sensitive to HSV-1 infection and infection was lethal with wild type virus (Pasieka, Cilloniz et al. 2009).

5. Conclusions

The aim of this thesis was to try and expand our knowledge in the inflammatory response of the eye to recurrent HSV infection. The role of TLRs is well studied in immune responses to infection and studies have been done to examine TLRs in the corneas of HSK patients compared with healthy controls. Results from these studies suggested that TLR 4, 8 and 9 were implicated in the pathogenesis of active HSV infection in the cornea whereas TLR7 may play a key role in HSK (Jin, Qin et al. 2007). For our study we examined the peripheral cells and their immune response. The majority of studies on inflammation, cytokines, TLRs and signalling pathways have been done either in murine models or in cell culture. But a study done in the Institute of Ophthalmology in Mexico measured the expression of intracellular IFN-γ and IL-4 in HSK patients before and after treatment with Acyclovir and dialyzable Leukocyte Extracts (DLE). This showed an increase in IFN-γ and a decline in IL-4 after treatment. These results were presented at the 13th International Congress of Immunology but not published.

In our study we wanted to examine the immune responses of patients suffering from HSK before and after treatment and also compare these findings with healthy controls. Initially we saw higher CD69 expression on $CD8^+$ T cells, $CD4^+$ T cells and NK cells. The over expression of this activation marker was expected, especially as T lymphocytes are recruited to sites of viral infection. This confirmed that we were dealing with infected cells. CD80 was also over expressed on B cells and MHC Class I and II were decreased on macrophages. This observation may be significant as Herpes Simplex Virus Type 1 has been shown to target the MHC Class II processing pathway for immune evasion (Neumann, Eis-Hubinger et al. 2003). Having looked at the cell activation markers cytokine profiles showed that TNFα was produced in greater quantities to healthy controls when we stimulated the TLR3 pathway. It also showed that TNFα production was greater in TLR4 stimulated cells in the inactive patients suggesting that TNFα plays a part in the initial inflammation but this has a greater effect on the TRIM pathway over the MyD88 pathway. IL-6 which is also involved in early inflammation events in HSV infection gave normal responses except when TLR3 was stimulated suggesting that TLR3 and the TRIF adaptor is the main pathway that responds to

HSV-1 infection. Rantes production showed decreases in active controls compared to healthy donors for both TLR3 and TLR4 stimulated cells. As Rantes is produced through transcription factors NF-κβ and IRF-3 this further implicates HSV-1 as interfering with the TLR3/TRIF pathway.

In the serum samples IL-1 which is one of the most prominent cytokines produced after HSV-1 infection was upregulated and although some decrease was seen after treatment levels were still higher than in the active controls compared to healthy donors. This is perhaps due to the latent infection and the patients inefficiency at clearing the HSV infection. IL-1 production might be a target for therapies as it seems that it has a persistent effect in HSK. There was a trend towards a decrease in IL-12 production in patient serum. This may tie in with the increasing amounts of total stat1 as IL-12 stimulates the secretion of type II interferons which are produced from Stat1 phosphorylation and translocation to the nucleus. We might expect decreases in total Stat1 in active patients as type II interferons are produced for viral clearance. IRF3 levels which are produced when TLR3 is activated are decreasing as expected as IRF3 is transported to the nucleus to activate genes for IFN transcription. Whereas total Stat 1 is increasing upon TLR 3 stimulation which suggests that HSV is using the TLR3 immune response to interfere with the Stat/Jak pathway which in turn will interfere with Interferon function for viral clearance.

The results in this thesis would all benefit from increased patient numbers especially for results which showed observable trends that were considered to be deviating from the expected immune and cellular responses but where statistically the results were shown to be insignificant. Time and scope did not allow it but a more focused study on the TLR3 responses and the IRF3 and Stat production of interferons may also be warranted as this is the pathway that showed results that indicated that it may be compromised in HSV-1 infection in the eye when HSK patients peripheral mononuclear cells were analysed.

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7. Appendices

Appendix i

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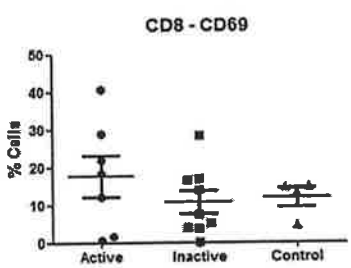

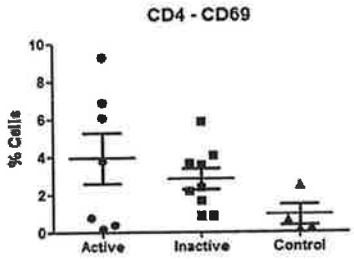

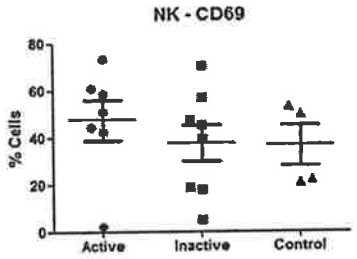

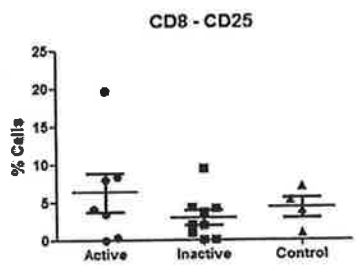

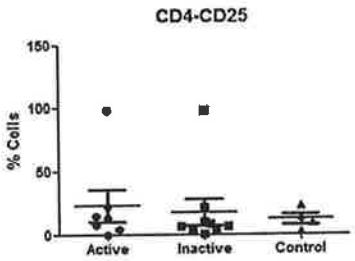

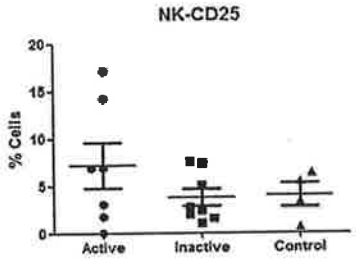

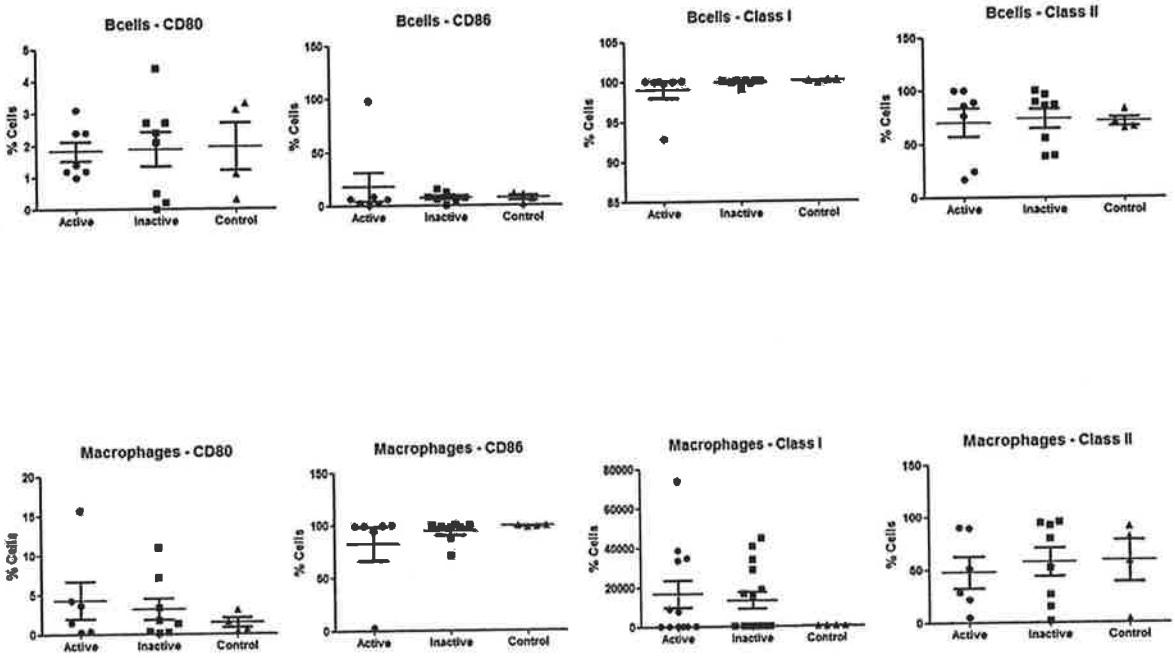
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Bcells - CD86
Bcells - Class I
Bcells - Class II
Macrophages - CD80
Macrophages - CD86
Macrophages - Class I
Macrophages - Class II
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Control

Appendix ii

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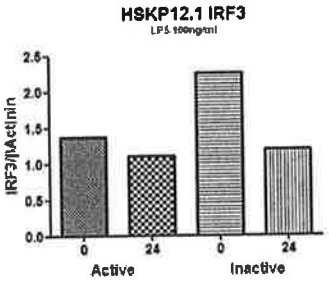

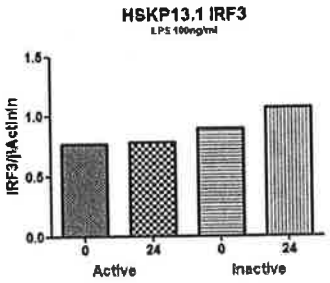

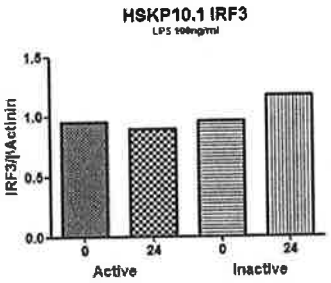

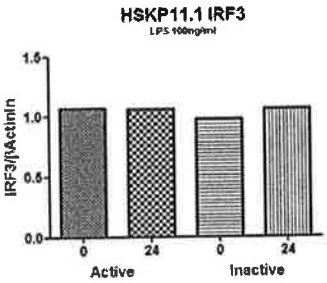

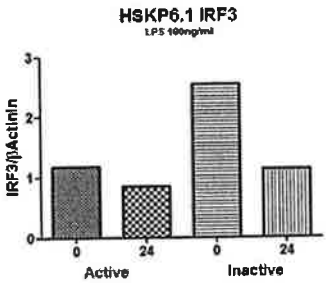

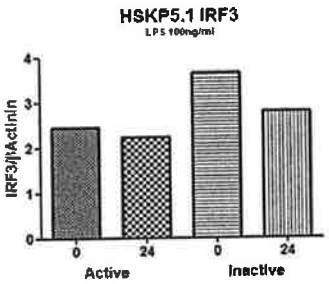

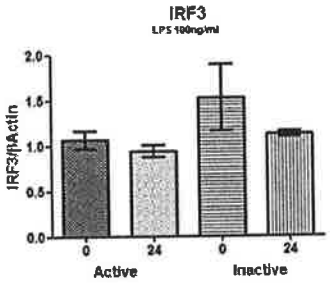

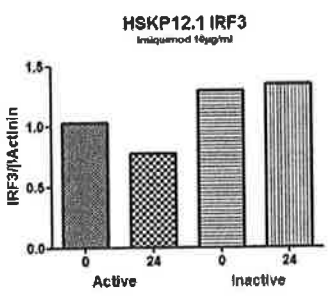
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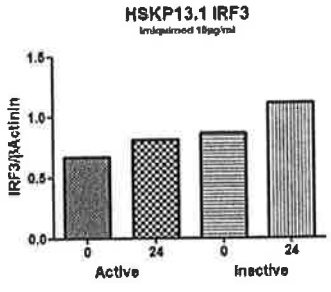
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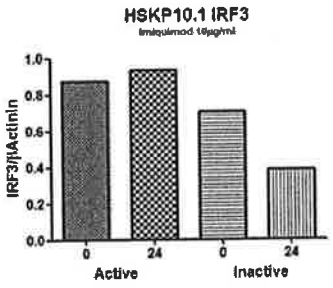
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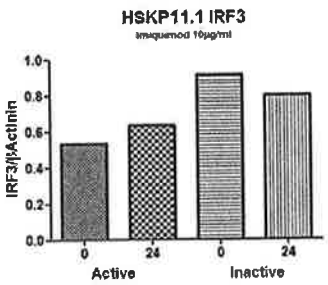
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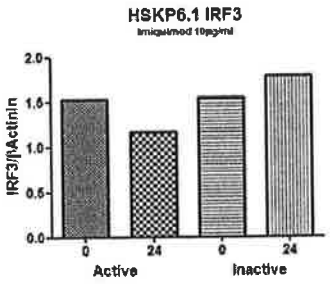
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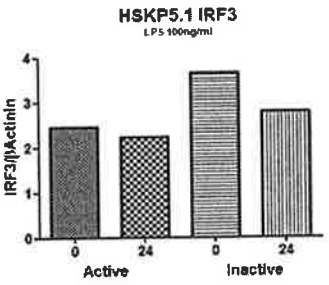
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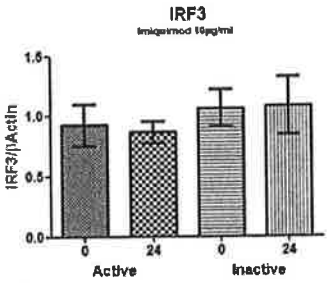
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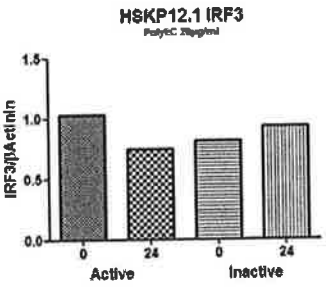
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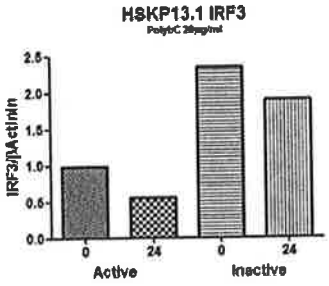
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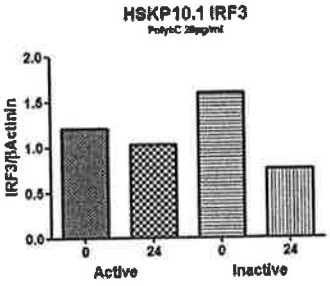
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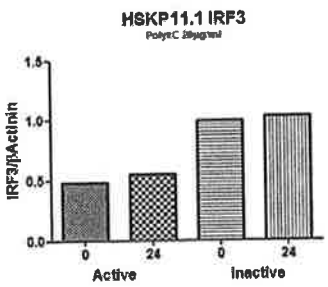
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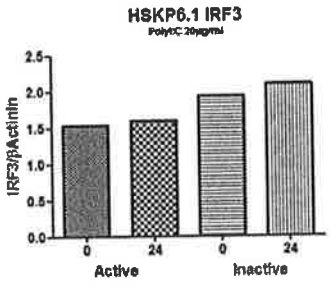
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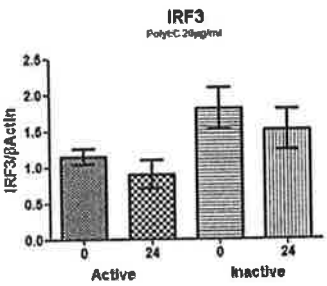
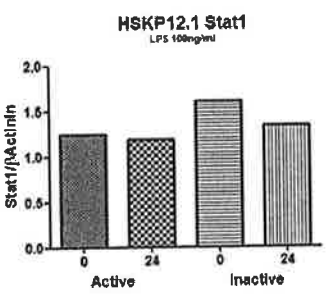
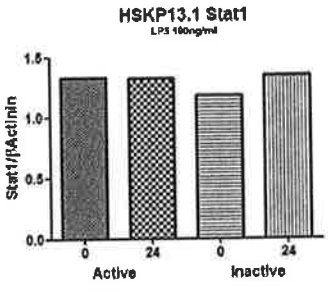
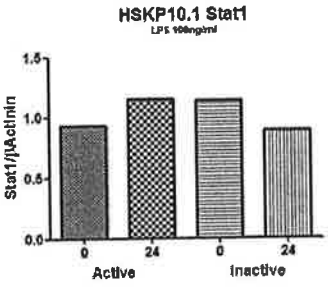
IRF3/βActinin
2.5
2.0
1.5
1.0
0.5
0.0
0
24
0
24
Active
Inactive

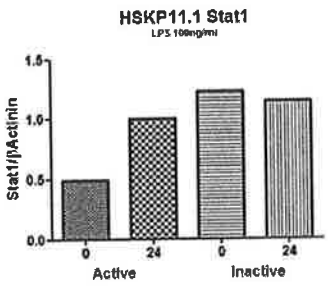

IRF3
IRF3/βActin
2.5
2.0
1.5
1.0
0.5
0.0
0
24
0
24
Active
Inactive

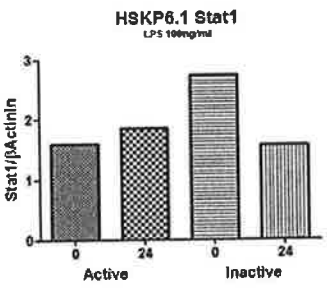
FACS individual patient graphs Stat1

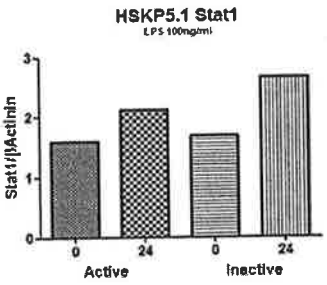

HSKP12.1 Stat1
LPS 100ng/ml
Stat1/βActinin
2.0
1.5
1.0
0.5
0.0
0
24
0
24
Active
Inactive

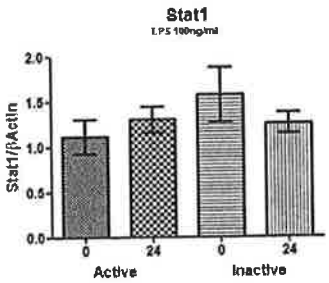

HSKP13.1 Stat1
LPS 100ng/ml
Stat1/βActinin
1.5
1.0
0.5
0.0
0
24
0
24
Active
Inactive

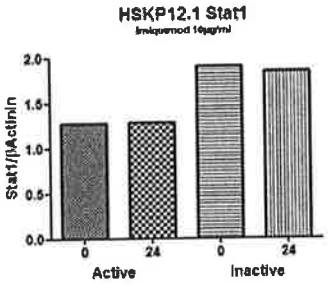

HSKP10.1 Stat1
LPS 100ng/ml
Stat1/βActinin
1.5
1.0
0.5
0.0
0
24
0
24
Active
Inactive

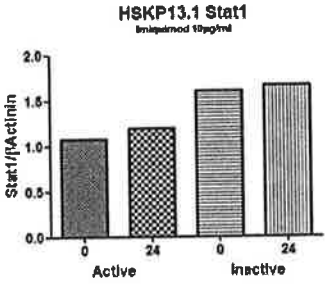

HSKP11.1 Stat1
LPS 100ng/ml
Stat1/βActinin
1.5
1.0
0.5
0.0
0
24
0
24
Active
Inactive

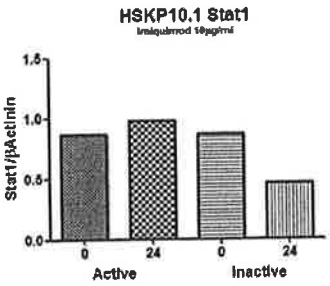

HSKP6.1 Stat1
LPS 100ng/ml
Stat1/βActinin
3
2
1
0
0
24
0
24
Active
Inactive

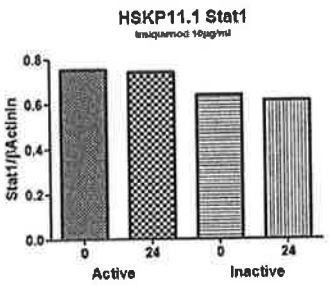

HSKP5.1 Stat1
LPS 100ng/ml
Stat1/βActinin
3
2
1
0
0
24
0
24
Active
Inactive

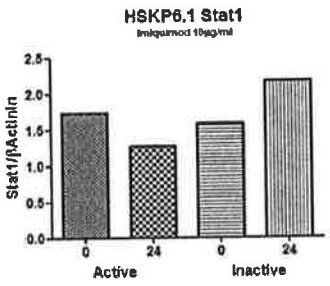

Stat1
LPS 100ng/ml
Stat1/βActin
2.0
1.5
1.0
0.5
0.0
0
24
0
24
Active
Inactive

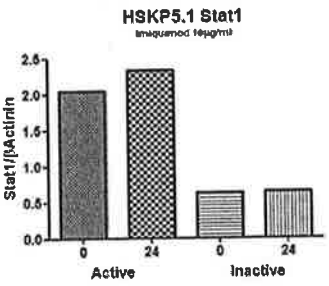

HSKP12.1 Stat1
Imiquimod 10µg/ml
Stat1/βActinin
2.0
1.5
1.0
0.5
0.0
0
24
0
24
Active
Inactive

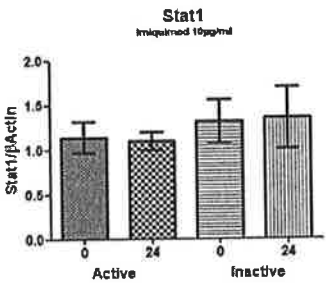

HSKP13.1 Stat1
Imiquimod 10µg/ml
Stat1/βActinin
2.0
1.5
1.0
0.5
0.0
0
24
0
24
Active
Inactive

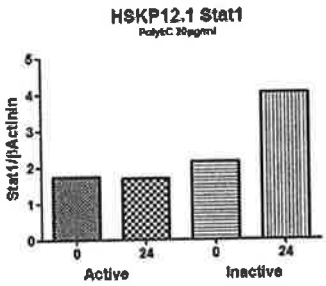

HSKP10.1 Stat1
Imiquimod 10µg/ml
Stat1/βActinin
1.5
1.0
0.5
0.0
0
24
0
24
Active
Inactive

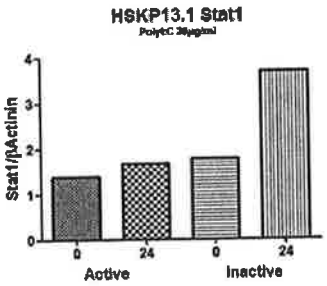

HSKP11.1 Stat1
Imiquimod 10µg/ml
Stat1/βActinin
0.8
0.6
0.4
0.2
0.0
0
24
0
24
Active
Inactive

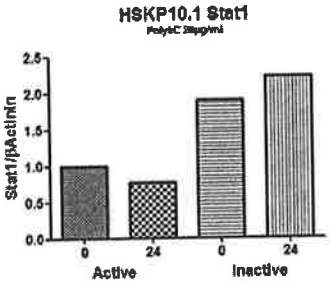

HSKP6.1 Stat1
Imiquimod 10µg/ml
Stat1/βActinin
2.5
2.0
1.5
1.0
0.5
0.0
0
24
0
24
Active
Inactive

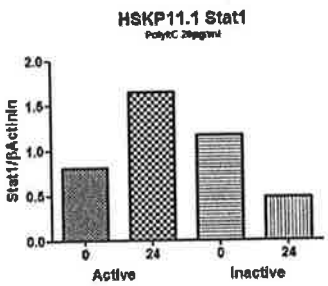

HSKP5.1 Stat1
Imiquimod 10µg/ml
Stat1/βActinin
2.5
2.0
1.5
1.0
0.5
0.0
0
24
0
24
Active
Inactive

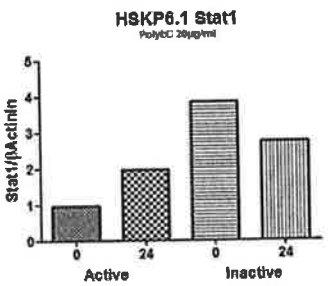

Stat1
Imiquimod 10µg/ml
Stat1/βActin
2.0
1.5
1.0
0.5
0.0
0
24
0
24
Active
Inactive

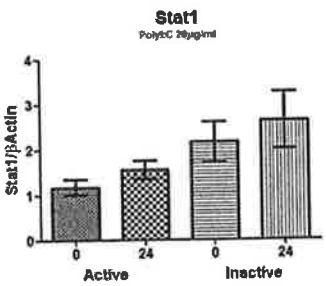

HSKP12.1 Stat1
Stat1/βActinin
0
24
0
24
Active
Inactive


HSKP13.1 Stat1
Stat1/βActinin
0
24
0
24
Active
Inactive


HSKP10.1 Stat1
Stat1/βActinin
0
24
0
24
Active
Inactive

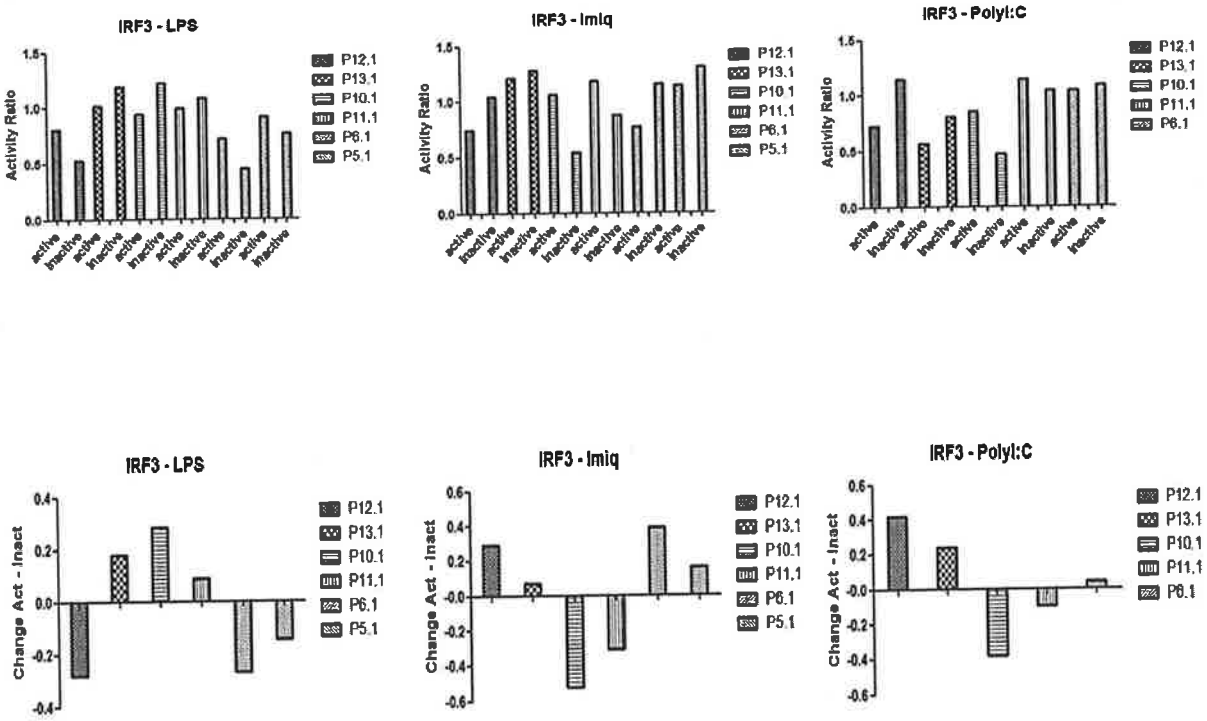

HSKP11.1 Stat1
Stat1/βActinin
0
24
0
24
Active
Inactive


HSKP6.1 Stat1
Stat1/βActinin
0
24
0
24
Active
Inactive


Stat1
Stat1/βActin
0
24
0
24
Active
Inactive

Appendix iii

Densitometry Ratio graphs – IRF3



Densitometry Ratio graphs – Stat1